REMARKS

A. Status of the Claims

Claims 1-29, 31-33, 38-41, and 43-60 were pending in the case at the time of the filing of this Request for Continued Examination (RCE). Claims 30 and 34-37 have been previously canceled without prejudice or disclaimer. Claims 38-60 are newly canceled without prejudice or disclaimer. Claims 1, 18, 31, and 33 have been amended in the Amendment set forth herein. No new claims have been added. Thus, claims 1-29, and 31-33 are currently under consideration.

B. The Declaration of Dr. Clayman Supports Novelty and Nonobviousness of the Claimed Invention

Applicants herein submit the Declaration of Dr. Gary Clayman (Exhibit A; hereinafter "the Clayman Declaration") as further support for the novelty and nonobviousness of the claimed invention in view of the references previously cited by the Examiner. Dr. Clayman presented the PowerPoint presentation entitled "Clinical protocol for wild type p53 gene induction in premalignancies of squamous epithelium of the oral cavity via an adenoviral vector," ("PowerPoint Presentation," Exhibit 1). He has declared that he is the principal investigator of Human Gene Transfer Protocol #0101-445, entitled "Clinical Protocol for Wild-Type p53 Gene Induction in Premalignancies of Squamous Epithelium of the Oral Cavity via an Adenoviral Vector," as discussed on page 10 of the minutes of the Recombinant DNA Advisory Committee (RAC) dated March 8, 2001, U.S. Dept. of Health and Human Services (Exhibit 2). Clayman Declaration, ¶3.

One slide of his PowerPoint is entitled "Special Protocol Testing Summary Pre Treatment" and it indicates "HPV of microdissected lesion." Dr. Clayman has declared that he had originally planned to determine whether microdissected lesions were infected with human papillomavirus (HPV) because "I hypothesized that HPV infection of cells makes the cells less

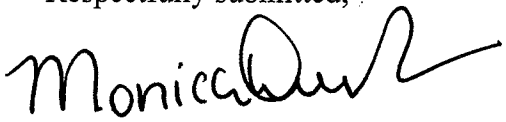
responsive to gene therapy with p53." Clayman Declaration, ¶4. This is because it was known that HPV expresses the E6 and E7 proteins, which can form specific complexes with tumor suppressor gene products. Clayman Declaration, ¶4, citing to Münger *et al.*, Cancer Surv. 1992; 12:197-217, 1992; Exhibit 3 of Clayman Declaration. In particular, he notes that it was known that HPV E6 protein can bind to p53 protein, which promotes the degradation of p53 protein. Clayman Declaration, ¶4. He has declared that "microdissection of lesions to assess for HPV status was not performed as part of the protocol because I had believed that the number of enrolled study participants was too low to allow for statistical evaluation concerning HPV status." *Id.* Therefore, Dr. Clayman concludes that "at the time of treatment, it was not known whether any patient was infected with HPV." Id.

Thus, the Declaration of Dr. Clayman supports Applicants' position that the claimed invention is novel over the Clayman powerpoint presentation and the RAC meeting minutes. Further, the Declaration of Dr. Clayman supports Applicants' position that the claimed invention is nonobvious over the RAC meeting minutes as evidenced by Oda and Flaitz.

C. Conclusion

It is respectfully submitted, in light of the above, that none of the pending claims are properly rejected. Reversal of the pending grounds for rejection is thus respectfully requested.

Respectfully submitted,



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Date: January 11, 2008

Monica A. De La Paz
Reg. No. 54,662
Attorney for Applicants

EXHIBIT A

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: George H. Yoo Serial No.: 10/747,798 Filed: December 29, 2003 For: p53 TREATMENT OF PAPILLOMAVIRUS AND CARCINOGEN-TRANSFORMED CELLS IN HYPERPLASTIC LESIONS	Confirmation No. 1871 Group Art Unit: 1633 Examiner: Scott D. Priebe Atty. Dkt. No.: INRP:104US

DECLARATION OF DR. GARY CLAYMAN

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 23313-1450

I, Gary Clayman, declare that:

1. I am a U.S. citizen residing at 6353 Westchester St., Houston, Texas. I am employed by the University of Texas, where I hold the title of Professor of Surgery and Chairman of the Department of Head and Neck Surgery of M.D. Anderson Cancer Center.

2. I am listed as an inventor on several patent applications that I understand are exclusively licensed to Introgen Therapeutics, Inc. I understand that Introgen is exclusively licensing the above-referenced case. I was previously unaware of this application prior to being told that my work was being cited as art.

A: 1657420
A: 229896(4X#0011.DOC)

3. I presented the PowerPoint presentation entitled "Clinical protocol for wild type p53 gene induction in premalignancies of squamous epithelium of the oral cavity via an adenoviral vector," ("PowerPoint Presentation," Exhibit 1). I am the principal investigator of Human Gene Transfer Protocol #0101-445, entitled "Clinical Protocol for Wild-Type p53 Gene Induction in Premalignancies of Squamous Epithelium of the Oral Cavity via an Adenoviral Vector," as discussed on page 10 of the minutes of the Recombinant DNA Advisory Committee (RAC) dated March 8, 2001, U.S. Dept. of Health and Human Services (Exhibit 2).

4. One slide of my PowerPoint is entitled "Special Protocol Testing Summary Pre Treatment" and it indicates "HPV of microdissected lesion." I had originally planned to determine whether microdissected lesions were infected with human papillomavirus (HPV) because I hypothesized that HPV infection of cells makes the cells less responsive to gene therapy with p53. This is because it was known that HPV expresses the E6 and E7 proteins, which can form specific complexes with tumor suppressor gene products (this is reviewed in Münger *et al.*, Cancer Surv. 1992; 12:197-217, 1992; Exhibit 3). In particular, it was known that HPV E6 protein can bind to p53 protein, which promotes the degradation of p53 protein. However, microdissection of lesions to assess for HPV status was not performed as part of the protocol because I had believed that the number of enrolled study participants was too low to allow for statistical evaluation concerning HPV status. Therefore, at the time of treatment, it was not known whether any patient was infected with HPV.

5. I declare that all statements made herein of our own knowledge are true, and that all statements of my own belief are believed to be true, and further that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this patent, and any reexamination certificate issuing thereon.

1/8/09
Date

Dr. Gary Clayman

A: 1657420
A: 239696(4X#0011.DOC)

EXHIBIT 1

Clinical Protocol for Wild Type p53 Gene Induction in Premalignancies of Squamous Epithelium of the Oral Cavity via an Adenoviral Vector

Primary Investigator: Gary Clayman M.D.

Sponsor: Introgen Therapeutics, Inc.

INTROGEN

RPR/INGN 201 (Ad5CMV-*p53*)

- Adenoviral vector expressing p53
- Completely sequenced
- CGMP Manufacturing in validated clean-room facility by experienced personnel
- Final product testing in compliance with CGMP and current industry practice

INTROGEN

Previous Human Exposure to RPR/INGN 201 (Ad5CMV-*p53*)

- In use in human clinical trials since 1995
- Over 480 people exposed by various routes of administration
- Head & Neck phase III trials actively ongoing
- Phase I & II trials ongoing in additional indications
- Large safety database for this material

INTROGEN

Protocol Description

- Combination of intramucosal injection of RPR/INGN 201 (Ad5CMV-*p53*) in area of lesion followed by a series of oral swishes with same
- Patients participate in protocol for 6 months, each 1 month cycle begins with 5 days of exposure to RPR/INGN 201 (Ad5CMV-*p53*)

INTROGEN

Protocol Description: Day 1

- Lesion will be injected with RPR/INGN 201 (Ad5CMV-*p53*)
- Biopsy of lesion and contralateral region after 2 hours
- 10% acetic acid rinse for 2 minutes
- RPR/INGN 201 (Ad5CMV-*p53*) swish for 30 minutes

INTROGEN

Protocol Description: Day 2-4

- 10% acetic acid rinse
- RPR/INGN 201 (Ad5CMV-*p53*) swish and spit
- Minimum 2 hour observation
- Repeat rinse and swish/spit

INTROGEN

Protocol Description: Day 5

- 10% acetic acid rinse
- RPR/INGN 201 (Ad5CMV-*p53*) swish and spit
- Minimum 2 hour observation
- Biopsy of lesion and contralateral region
- Repeat rinse and swish/spit

INTROGEN

Special Protocol Testing Summary
Pre Treatment

- p53 genotype of microdissected lesion
- HPV of microdissected lesion
- H&E on all biopsy specimens
- TUNEL on lesion & contralateral region
- CAR on lesion & contralateral region
- p53 IHC on contralateral region only
- Antibody to serotype 5 adenovirus in serum

INTROGEN

Special Protocol Testing Summary Post Treatment

- H&E on lesion & contralateral region
- TUNEL lesion & contralateral region
- CAR lesion & contralateral region
- p53 IHC on contralateral region

INTROGEN

RAC Reviewer Protocol Questions

- Risk to benefit ratio
- Efficacy evaluations
- Safety of acid rinse
- 30 minute duration of swish
- Consenting process

INTROGEN

Risk to benefit ratio

INTROGEN

Disease Characteristics

- Patients diagnosed with a preneoplastic lesion of the oral cavity will progress to a malignant state, most within six months.
- Surgery is frequently not effective because of diffuse, multifocal nature of these lesions.
- Patients eligible for this trial will have failed other approaches being tested.

INTROGEN

Why this study, Why this patient population

- Two Case Studies

INTROGEN

Case Study #1

- Prior history of multiple surgical procedures for removal of retromolar area lesions
- 1986 diagnosis mild dysplasia in multiple locations in oral cavity
- 1988 treatment with topical Retin A gel, discontinued because of patient discomfort

INTROGEN

Case Study #1 (continued)

- Aug 1993 carcinoma in situ, surgically removed, margins pathologically free of cancer
- Nov 1993 moderate to severe dysplasia in same area, lesion removed by laser ablation

INTROGEN

Case Study #1 (continued)

- Feb 1994 moderate dysplasia in same area
- July 1994 continued inflammation & dysplasia, Biochemoprevention therapy
- Dec 1994 continued inflammation & dysplasia

INTROGEN

Case Study #1 (continued)

- April 1996 Invasive squamous carcinoma
- May 1996 Resection of major portions of oral cavity, margins pathologically free of cancer
- May 1996 Post operative radiation therapy
- July 1996 Extensive local, facial and infratemporal fossa recurrence treated with chemotherapy and radiotherapy
- Sep 1996 Patient died of disease

INTROGEN

Case Study #2

- June 1992 Premalignant left oral lesion surgically removed, recurrence within six months
- Feb 1994 Laser ablation to remove left oral tongue premalignant lesion, recurrence within six months
- May 1996 biopsy shows mild to moderate dysplasia + hyperkeratosis of left oral tongue
- July 1996 Biochemoprevention studies initiated

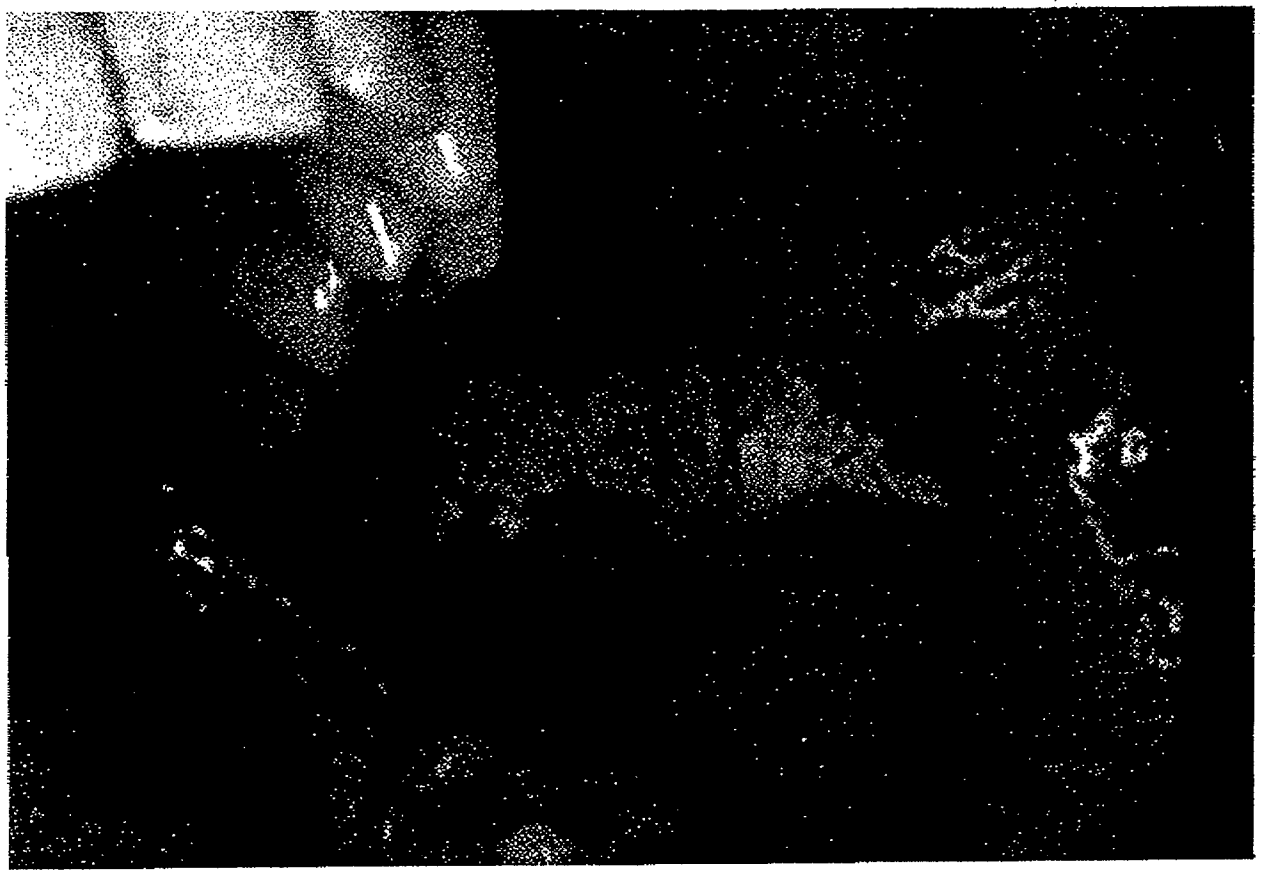
INTROGEN

Case Study #2 (continued)

- Jan 1997 continued left oral tongue dysplasia
- Dec 1997 new lesion on right oral tongue; surgery to remove part of tongue, floor of mouth and neck. Laser ablation to clear a larger area. Margins free. Patient ineligible for biochemoprevention since he developed lesion while on that attempted prevention approach
- Feb 2001 Patient has dysplasia in two areas of oral cavity

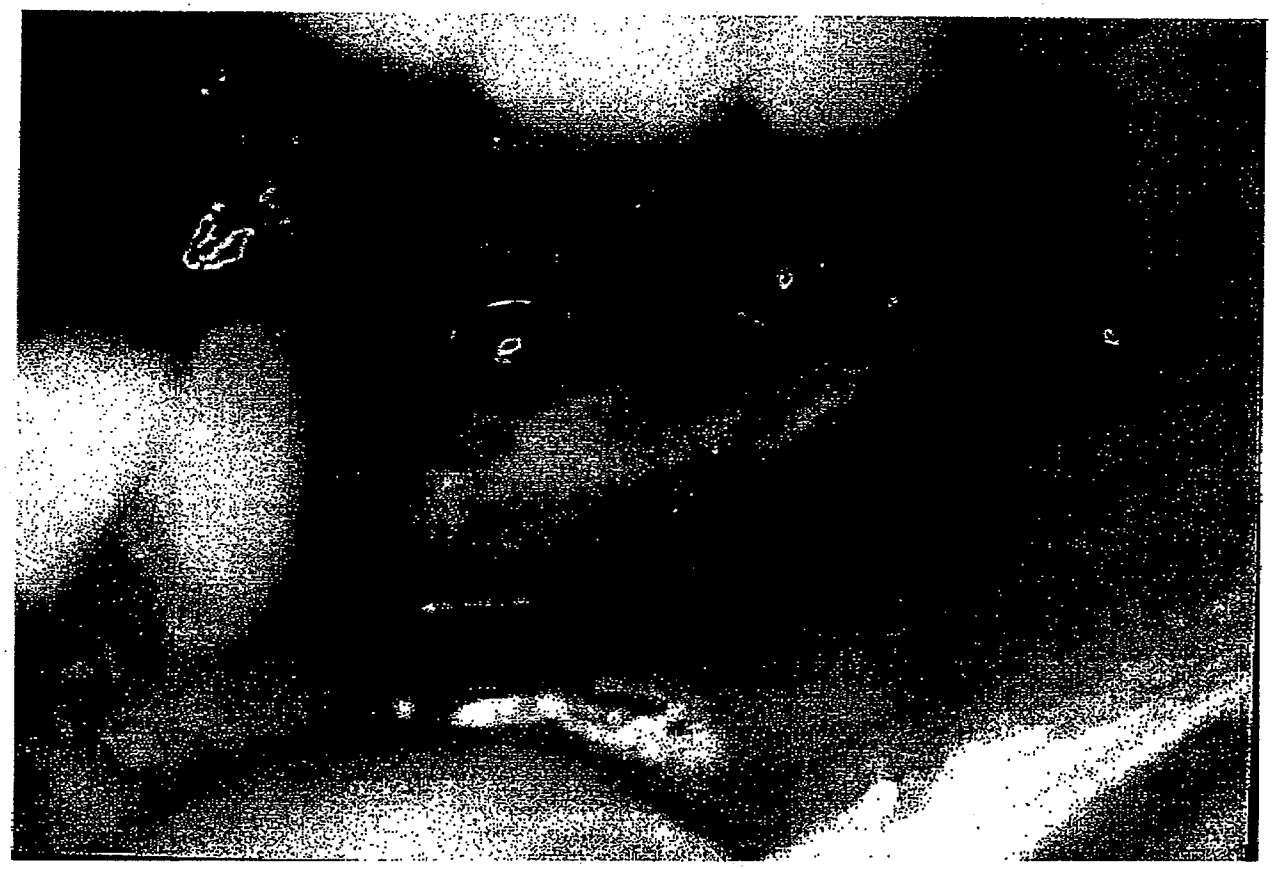
INTROGEN

Diagnosis: Leukoplakia without dysplasia of the Oral Cavity



INTROGEN

Diagnosis: Erythroplasia with severe dysplasia of the Oral Cavity



INTROGEN

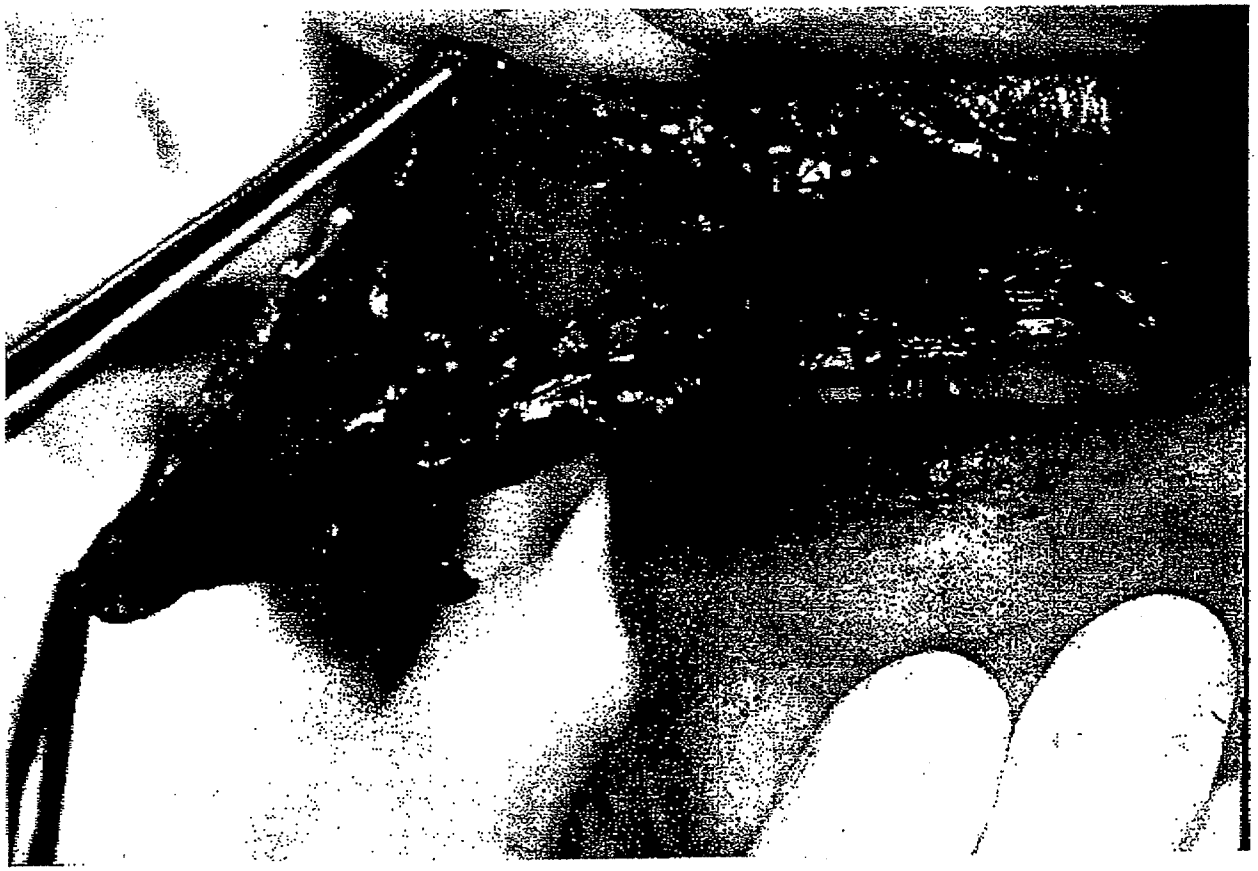
Diagnosis: Preneoplastic Lesion of the Oral Cavity

INTROGEN

Progression to Malignant Lesion

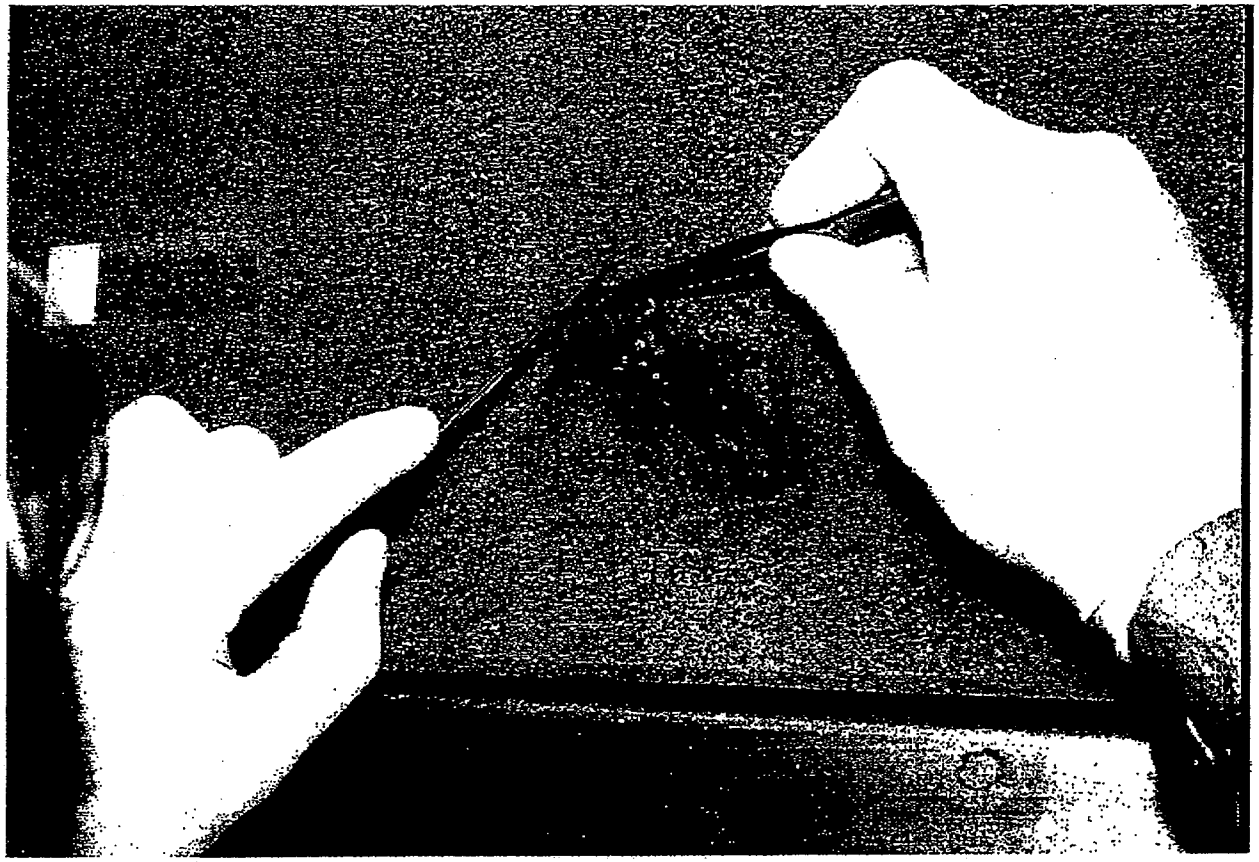
INTROGEN

Surgical removal of carcinoma *in situ*



INTROGEN

Comprehensive pathologic analysis of premalignant lesion



INTROGEN

Extensive surgical margin required for premalignant lesion

INTROGEN

Efficacy Evaluation versus Protocol Goals for a Phase I/II Clinical Trial

- As with all Phase I/II clinical trials, the primary objective is to evaluate safety
- Any scientific information gathered is a secondary objective
- Any efficacy information that comes from a phase I/II is a secondary objective

INTROGEN

Previous Routes of Administration for RPR/INGN 201 (Ad5CMV-*p53*)

- Intratumoral in Head & Neck
- Lavage in Lung
- Intratumoral in Lung
- Intratumoral in prostate, ovary, breast, bladder
- IV

INTROGEN

Safety of Acid Rinse

- 10% acetic acid approximately the strength of household vinegar (6%)

INTROGEN

pH Comparison

Item	FDA or USP acceptable pH range	Example of tested pH
10% acetic acid	N/A	2.2
Coca Cola	N/A	2.3
Sauerkraut	3.4-3.6	3.3
Grapefruit juice	3.0-3.3	3.5

INTROGEN

Questions on
30 minute duration of swish

- Route of administration
- Safety for other tissues
- Potential for aspiration

INTROGEN

30 minute duration of swish

- Common route of administration
- Used for Tingel, approved flouride compound
- Not uncomfortable for patients
- Will be performed under clinical observation

INTROGEN

Previous Routes of Administration to Head & Neck

- Intratumoral and intramucosal injections in multiple head and neck sites
- Intraoperative injection and lavage into surgical beds
- Intraoperative injection into mucosal margins following cancer removal

INTROGEN

Previous of Administration to Lung

- Bronchoalveolar lavage in Lung
- Intratumoral in Lung

INTROGEN

Additional Previous Routes of Administration

- Intraprostatic injection
- Intervesicle in bladder
- Intravenous
- Intraperitoneal in ovary
- Intratumoral in breast

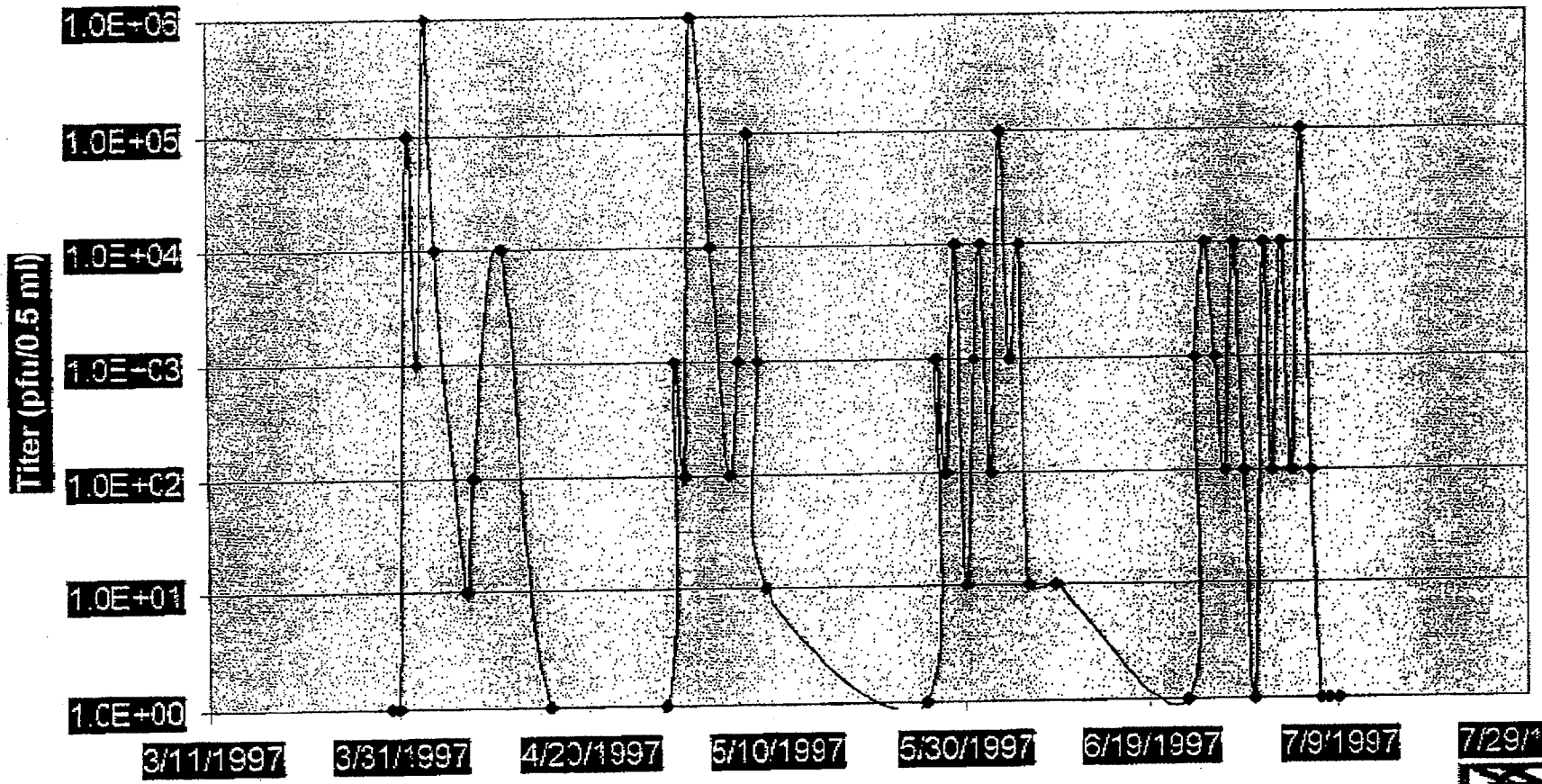
INTROGEN

RPR/INGN 201 (Ad5CMV-*p53*): Safe for normal tissue?

- Not toxic to non-malignant structures
- Post-surgical injection into healthy surgical beds of the Head and Neck and oral cavity
- Intraprostatic injection exposed normal prostate tissue
- Lavage of cerebral cortex exposed normal brain tissue
- All routes tested thus far are well tolerated

INTROGEN

RPR/INGN 201 in Upper Aerodigestive Tract Secretions Following Intratumoral Administration



INTROGEN

Post surgical injection into healthy surgical bed

INTROGEN

Informed Consent

- Viewed by investigator and sponsor of this trial as a process beyond obtaining appropriate signature
- Witness required by IRB to observe the informed consent process
- Important gene transfer considerations included

INTROGEN

EXHIBIT 2

RECOMBINANT DNA ADVISORY COMMITTEE

Minutes of Meeting

March 8, 2001

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

CONTENTS

Note: The latest Human Gene Transfer Protocol List can be found at the Office of Biotechnology Activities' Web site at <http://www4.od.nih.gov/oba/RDNA.htm>.

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
NATIONAL INSTITUTES OF HEALTH
RECOMBINANT DNA ADVISORY COMMITTEE
MINUTES OF MEETING[1]
March 8, 2001

The Recombinant DNA Advisory Committee (RAC) was convened for its 81st meeting at 8:30 a.m. on March 8, 2001, at the National Institutes of Health (NIH), Building 31, Sixth Floor, Conference Room 10, 9000 Rockville Pike, Bethesda, MD 20892. Dr. Claudia A. Mickelson (Chair) presided. In accordance with Public Law 92-463, the meeting was open to the public. The following individuals were present for all or part of the meeting:

Committee Members

C. Estuardo Aguilar-Cordova, Harvard Gene Therapy Initiative
Dale G. Ando, Cell Genesys
Xandra O. Breakefield, Massachusetts General Hospital
Louise T. Chow, University of Alabama, Birmingham
Theodore C. Friedmann, University of California, San Diego
Jon W. Gordon, Mount Sinai School of Medicine
Jay J. Greenblatt, National Cancer Institute, National Institutes of Health
Eric T. Juengst, Case Western Reserve University
Nancy M. P. King, University of North Carolina, Chapel Hill
Sue L. Levi-Pearl, Tourette's Syndrome Association
Ruth Macklin, Albert Einstein College of Medicine
M. Louise Markert, Duke University Medical Center
Claudia A. Mickelson, Massachusetts Institute of Technology

Executive Secretary

Amy P. Patterson, National Institutes of Health

Ad Hocs/Speakers

Andrew George Braun, Harvard Medical School
Boro Dropulic, VIRxSYS
Cynthia Dunn, Clinical Research Institute
John J. Fung, University of Pittsburgh
Carter Van Waes, National Institute on Deafness and Other Communication Disorders, NIH

Nonvoting/Agency Representatives

Kristina C. Borror, Office for Human Research Protections, Department of Health and Human Services
Philip Noguchi, U.S. Food and Drug Administration

National Institutes of Health Staff Members

Sarah Carr, OD
Janita Coen, NHLBI
J.R. Dixon, OD
Kelly Fennington, OD

---

[1] The Recombinant DNA Advisory Committee is advisory to the National Institutes of Health (NIH), and its recommendations should not be considered as final or accepted. The Office of Biotechnology Activities should be consulted for NIH policy on specific issues.

Joseph F. Gallelli, CC
Robert Jambou, OD
Kathryn R. Lesh, OD
Barbara McDonald, OD
Cheryl McDonald, OD
Marina O'Reilly, OD
Alexander Rakowsky, OD
Gene Rosenthal, OD
Thomas Shih, OD
Allan Shipp, OD
Sonia I. Skarlatos, NHLBI
Lana Skirboll, OD

Others

Approximately 45 individuals attended this 1-day RAC meeting. A list of attendees appears in Attachment II.

I. Call to Order and Opening Remarks/Dr. Mickelson

Dr. Mickelson, RAC Chair, called the meeting to order at 8:30 a.m. on March 8, 2001. Notice of this meeting under the *NIH Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines)* was published in the *Federal Register* on February 23, 2001 (66 FR 11305). The agenda included reviews of two gene transfer protocols, data management, the proposed action to amend the *NIH Guidelines* requirements for reporting and analysis of serious adverse events, a proposed plan for addressing issues related to the roles and responsibilities of Institutional Biosafety Committees, a lentiviral vector system under development for use in clinical trials, risk-containment practices for strain B of the common bacterium *Escherichia coli (E. coli)* frequently used for large-scale production processes, and the U.S. Food and Drug Administration's (FDA) proposed disclosure rule, "Availability for Public Disclosure and Submission to FDA for Public Disclosure of Certain Data and Information Related to Human Gene Therapy or Xenotransplantation."

Following a review of conflict-of-interest rules, Dr. Mickelson offered a brief summary of the March 7, 2001 Fourth National Gene Transfer Safety Symposium: Safety Considerations in the Use of AAV Vectors in Gene Transfer Clinical Trials. Several RAC members noted that this symposium was an example of how regulatory and review bodies can respond quickly to an ongoing concern within the scientific community. The Office of Biotechnology Activities (OBA) was congratulated for organizing the symposium and putting together an effective program, and Mark S. Sands, Ph.D., Washington University School of Medicine, was lauded for generating awareness among the scientific community and the public of the issues raised by his preclinical research results.

II. Minutes of the December 13 and 15, 2000 Meeting/Drs. Gordon and Juengst

Dr. Gordon noted that a few technical words were misspelled, and he provided a copy of the minutes that included those corrections.

A. Committee Motion 1

As moved by Dr. Gordon and seconded by Dr. Markert and with the understanding that any misspellings will be corrected, the RAC unanimously approved the December 13 and 15, 2000 minutes by a vote of 12 in favor, 0 opposed, and 0 abstentions.

III. Discussion of Human Gene Transfer Protocol #0101-443: Evaluation of the Safety and Effects of *ex vivo* Modification and Reinfusion of CD34+ Cells by an Antisense Construct Against HIV-1 in a Retroviral Vector

Principal Investigator:	Jeffrey C. Laurence, M.D., Weill Medical College, Cornell University
Other Investigators:	Marcus A. Conant, M.D., Dermatology/HIV Consultant;
	Dean L. Engelhardt, Ph.D., Enzo Therapeutics;
	Barbara E. Thalenfeld, Ph.D., Enzo Therapeutics
Sponsor:	Enzo Therapeutics, Inc.
RAC Reviewers:	Dr. Aguilar-Cordova, Ms. King, and Drs. Markert and Mickelson
Ad Hoc Reviewer:	John J. Fung, M.D., Ph.D., University of Pittsburgh

A. Protocol Summary

Investigators have demonstrated that the growth of HIV-1 can be blocked by the use of antisense genes. Three independent antisense sequences directed against 2 HIV-1 functional regions, tar and tat/rev, have been embedded into separate cloned human U1 RNA genes. This triple U1/HIV-1 antisense cassette was incorporated into a Moloney murine leukemia virus derived vector (HGTV43) used to transduce CD34+ cells. Preclinical data suggested that the presence of the anti-HIV-1 genetic antisense RNA in CD4+ cells would be sufficient to manage HIV-1 levels in infected subjects.

A phase I clinical trial was initiated in which peripheral blood stem cells (PBSC) from HIV-1 infected research participants were transduced with HGTV43 and reinfused. Results from the clinical protocol demonstrate long term (6-12 months) survival of antisense RNA in a low number of bone marrow stem cells as well as in the peripheral blood mononuclear cells (PBMC) and CD4+ fraction. Since this low number of transduced PBMC has remained constant over a number of months, these data support the conclusion that stable engraftment of some of the antisense RNA-producing PBSC has occurred. Finally, there is no evidence that multiple infusions led to increased levels of engraftment.

This protocol is a continuation of the trial reported above. The investigators propose to increase the number of CD4+ cells producing anti-HIV-1 genetic antisense RNA. The investigators propose to isolate a population of PBSC from HIV-1 positive participants previously treated with G-CSF. After this isolation, the participants will receive a treatment of immune conditioning using mycophenolate mofetil (MMF). The PBSC will be transduced with HGTV43. After the transduction is complete, the participants will be irradiated in an outpatient procedure (600cGy, TBI), and the transduced cells containing the antisense genes will be re-infused into the HIV-1 participant. The end points of this study are the safety of the procedure and the extent of engraftment and proliferation of the engineered cell population. The study will enroll up to 6 participants.

B. Written Comments From Preliminary Review

Seven RAC members recommended that the protocol warranted public discussion. Ms. King and Drs. Markert and Mickelson submitted written reviews, as did *ad hoc* reviewer Dr. Fung, to which the investigators responded in writing and during this meeting.

Dr. Aguilar-Cordova raised a concern about the potential for high risk to the participants if radiation increased viral load simultaneous with reducing the immune response. He also asked which chemotherapeutic agent would be used and its effect on HIV Long Terminal Repeat (LTR) expression. Another issue of concern was the stability of the integrated vector. The vector contains three repeat sequences which increase the potential for recombination. He requested that the investigators provide more information about the packaging cell line.

In order to calculate the risk/benefit ratio, Ms. King requested more information on the effects of total body irradiation (TBI): potential for improved engraftment vs. immune suppression. Regarding the informed consent document, Ms. King noted that the potential benefit to participants was overstated, the statement of the risk of bone marrow suppression should be emphasized, an autopsy request should be included, an

appropriate financial disclosure statement should be added, and the document should be rewritten in second person.

Dr. Markert asked about the effect of irradiation on existing peripheral T cells and the thymus since these are sources of T-cell renewal in adults. If the thymus is damaged prior to or during irradiation, the participant would be unable to regenerate functional T cells. She recommended that only participants with proper thymic function should be enrolled and that, if the first two participants' subsequent test results show the thymus has been damaged such that T cells cannot be made, the study should be halted. She asked that the vector facility be properly audited to ensure that Good Manufacturing Procedures are followed, that procedures be performed on participants in a clinic rather than a physician's office, and that a Data and Safety Monitoring Board (DSMB) be established.

Dr. Mickelson focused on concerns about whether the use of the proposed conditioning/ablation treatments would significantly increase the risks associated with trial participation for this patient population. She also questioned whether using TBI would increase the risk of neoplasia, or the rate of appearance of HIV variants, thus affecting the efficacy of concurrent drug therapies. Because of these possible effects, she asked why TBI was selected rather than high-dose chemotherapy. She also questioned the low efficiency of transduction of CD34+ cells and which differentiated cell types might express the antisense RNA after engraftment.

Dr. Fung expressed concerns about the lack of information in the preliminary data regarding multiple-dose subjects, the effect of TBI on HIV replication, the use of immunosuppressive agents in an autotransplant, and the use of human serum for the isolation of CD34+ cells.

C. RAC Discussion

Several issues were raised by RAC members during discussion in addition to those expressed by the initial reviewers:

- Dr. Markert asked why the researchers used fetal calf serum in this protocol.
- Dr. Friedmann expressed concern that the vector construct may be prone to genetic rearrangements.
- Ms. Levi-Pearl commented that the informed consent document did not disclose information regarding whether the investigators have financial interests with the sponsor.
- Dr. Markert suggested that investigators use an immunoscope prior to and after the procedure to provide useful immunologic information about thymic function. The DSMB could use these comparative data to keep track of subjects' immune status and to decide whether to halt the trial.

D. Investigator Response

Regarding the concern about administering product in a physician's office, Dr. Conant responded that his office is set up to respond to severe hypotension and other immediate reactions, and provides both nursing staff and appropriate equipment. He agreed with Dr. Markert's suggestion of starting the trial with two subjects and then asking a DSMB with immunology expertise to review those results before proceeding. As to testing subjects for thymic function, Dr. Conant expressed his belief that only a small percentage of patients would be excluded as a result of thymic dysfunction, but agreed to implement that test. In response to Dr. Mickelson's question about using radiation therapy instead of chemotherapy, he stated that chemotherapy would be more detrimental than the low-dose radiation therapy proposed for this trial.

Dr. Laurence stated that the level of radiation proposed is standard treatment at New York Hospital's transplant unit and has been approved for treating cancer patients who are HIV positive. In response to Dr. Fung's questions about the proposed immunosuppressive regimen, Dr. Laurence responded that it is a mild, immune-conditioning regimen and that MMF appears to be an effective anti-HIV agent that

synergizes with other anti-HIV drugs. Dr. Conant noted mycophenolic acid is also useful in arresting differentiation of the transduced CD34+ cells following readministration to the participant. Dr. Laurence also indicated that fetal calf serum would be replaced with human serum to avoid possible antibody formation.

In response to Dr. Aguilar-Cordova's suggestion to use Southern blot analysis on target clones to check for rearranged vectors in the transduced cells, Dr. Engelhardt stated that the investigators have assayed by amplification of the insert rather than performing Southern blots on transduced cells.

E. Public Comment

No public comments were offered.

F. RAC Recommendations

Dr. Mickelson summarized the RAC recommendations as follows:

- A DSMB should be used to review the thymic function data and other safety information to determine whether the study should continue or what the next steps should be. At least one immunologist should be involved as immunologic adverse events may well occur. For research subject safety, this expert input on the review of safety data and protocol design is important.
- Southern Blot analysis should be conducted to assess vector construct stability in both the vector producing cells and the transduced cells.
- With regard to the informed consent document, the partial ablation radiation and pre-conditioning regimen should be clarified and this explanation should be moved forward to a more prominent position in the document. Also, the informed consent document should disclose whether the investigators have financial interests in Enzo Therapeutics, Inc.
- In order to diminish the host immune rejection response, xenoproteins such as fetal calf serum should be replaced with human or autologous proteins where possible.

G. Committee Motion 2

It was moved by Dr. Aguilar-Cordova and seconded by Dr. Markert that the recommendations expressed the views of the RAC and, following review by the RAC members and ad hoc reviewers, would be reiterated in a letter to the investigators and sponsor. The vote was 13 in favor, 0 opposed, and 0 abstentions.

IV. Optimization of HIV-1 Vectors Containing an Anti-HIV Antisense Payload for Gene Transfer into HIV-Infected Individuals/Boro Dropulic, Ph.D., VIRxSYS

Dr. Dropulic presented a lentiviral vector, VRX496, being developed for use in *ex vivo* gene transfer into HIV patients. The vector is derived from HIV-1, and does not encode any viral proteins. Expression of an antisense RNA to HIV-1 envelope is controlled by the HIV LTR, limiting expression only to HIV infected cells also expressing Tat and Rev. The clinical goal would be to interfere with wild type (wt)-HIV *in vivo* to decrease the viral load set point and to increase CD4 T-cell survival in order to postpone the development of acquired immune deficiency syndrome. Because the vector consists of only HIV-1 sequence, it may have safety advantages since it would introduce no new sequences into possible recombinants between the vector and wt-HIV-1; therefore, any replication-competent recombinants generated would not have the potential to be more pathogenic than wt-HIV.

In vitro results were presented showing the efficiency of human CD4 cell transduction and inhibition of HIV replication in challenged transduced cells. Biodistribution studies were performed in a mouse NOD/SCID model injected with transduced human T cells.

Dr. Dropulic also outlined the design of the clinical trial that is expected to be mounted. It would involve *ex vivo* transduction of CD4 T cells isolated from the research participant. Before re-administration, the cell product would be assayed for the presence of helper RNA or DNA. Research participants in this incremental dose-escalation trial would be monitored for differential viral load, CD4 count, and replication competent retrovirus (RCR).

A. RAC Discussion

Dr. Aguilar-Cordova asked why a vector that did not mobilize well was being pursued since mobilization should amplify the inhibitory effect while a vector without that capability would not have any advantage over MLV based retroviral vectors. Dr. Dropulic agreed that mobilizing vectors would have increased efficacy, but the vector was chosen for its safety features. He suggested that an HIV-1 derived vector would be more effective because the vector HIV RNA would track intra-cellularly to the sites of wt-HIV-1.

Dr. Mickelson and Dr. Friedmann asked about potential problems with toxicity or immune responses to the vector pseudotyped with the Vesicular Stomatitis Virus G (VSVG) envelope. Dr. Ando pointed out that because of the potential for recombination, the choice of envelope is not trivial and assays for RCR are limited in their sensitivity. Dr. Dropulic described the lot release criteria involving polymerase chain reaction (PCR) detection of helper RNA or DNA sequence and cell assays to look for any potential replication-competent virus.

Dr. Markert suggested that participants should be followed with an immunoscope to test for immune diversity. Dr. Dropulic responded that in the animal studies for which preparations are currently under way, immunoscopic analysis of the cells will be performed and cells will be tested by fluorescence-activated cell sorter for various receptors. Dr. Markert indicated that the new cytokine assays might result in allergies.

B. Public Comment

No public comments were offered.

V. Discussion of Risk-Group Designation for Strain B of *E. coli*/Drs. Ando and Mickelson

Dr. Ando explained that the University of Florida requested a definition of the risk-group classification for *E. coli* B strain be developed. Strain B is widely used in industry for fermentation and large-scale manufacturing of proteins because of the increased stability of cloned sequences compared with that of *E. coli* K-12.

Dr. Mickelson suggested that all *E. coli* strains could be placed into Risk Group 1 (RG1), nonpathogenic organisms, provided they lack virulence genes, contain deletions in metabolic genes so they are dependent on laboratory media, and do not make any known toxins. She explained that certain *E. coli* strains, such as K-12, are exempt from the *NIH Guidelines* because they meet a fourth criterion: inability to colonize the human gut. Rather than making decisions on a strain-by-strain basis, she suggested the generation of a general statement outlining the characteristics required for *E. coli* strains to be designated RG1 under the *NIH Guidelines*. Dr. Patterson indicated that a strawman proposal for this had been developed which could be put forward as a proposed action.

In the interim, a letter will be drafted in reply to the University of Florida's request that its strain of *E. coli* be considered RG1, as long as it does not contain toxins or virulence factors and there is metabolic dependence on laboratory media. Drs. Ando and Mickelson will work on the wording of the letter and distribute it to RAC members for review before sending.

Proposed language to amend the *NIH Guidelines* will be brought to the RAC at its next meeting and will then be published in the *Federal Register* for a public comment period. Dr. Mickelson offered the following general phrasing of the amendment language: If a strain can be shown not to produce any of the known bacterial toxins, does not contain any of the known major virulent factors for *E. coli*, and it carries

deletions in the metabolic genes that make it dependent on laboratory media, then those strains should be considered as Risk Group 1 *E. coli* for both large-scale and laboratory work.

A. Committee Motion 3

It was moved by Dr. Gordon and seconded by Dr. Markert that the strain of *E. coli* proposed by the University of Florida be considered Risk Group 1 and that draft language be developed to amend the *NIH Guidelines*. The vote was 12 in favor, 0 opposed, and 1 abstention.

VI. Proposed Action To Amend the *NIH Guidelines* Requirements for Serious Adverse Event Reporting (SAER): RAC Discussion and Vote/Dr. Macklin

Dr. Macklin called on Dr. Patterson and Mr. Allan Shipp, OBA.

A. Dr. Patterson

Dr. Patterson presented an overview of the Proposed Action that would amend the *NIH Guidelines* to enhance the reporting of safety information, its assessment, and its communication to the scientific community and the public. There are four elements to the proposal: (1) harmonization of the scope and timing of SAER to create one set of reporting criteria to both the NIH and FDA; (2) public access to safety information that will not be considered trade secret; (3) protection of research participant privacy in SAER; and (4) establishment of a national data assessment board. The Proposed Action establishes one set of reporting criteria for researchers to follow for both NIH and FDA and will provide enhanced and systematic analysis of safety data across all trials that will be presented publicly to inform about the design and conduct of ongoing and future clinical trials.

The proposed Gene Transfer Safety Assessment Board (GTSAB) would function as a mechanism for collecting, analyzing, and publicly reporting safety information across all trials. As such, it would facilitate early recognition of trends; report findings, conclusions, and aggregated trend analyses for public discussion at RAC meetings; and inform research participants, clinical investigators, basic scientists, Institutional Review Boards (IRBs) and Institutional Biosafety Committees (IBCs), and the public. The GTSAB would operate in an analytic and advisory capacity and would not supersede or replace the responsibilities of FDA or local review bodies in the day-to-day review of, and real-time response to, safety information. Approximately 15 members would make up this new Board, with outside experts in relevant fields constituting the majority of its membership; other members would include two RAC members, and NIH and FDA members. The board would meet quarterly in closed sessions prior to RAC meetings and provide reports to the RAC as well as publish periodic summary reports and cumulative trend analyses.

Dr. Patterson also reported on the status of the development of a national database for gene transfer clinical research. Using a controlled reporting vocabulary, this relational database will include product descriptors, elements of clinical trial design, and safety and toxicity data. It will be query capable and Web based. As an analytic tool for FDA, NIH, and advisory boards, this database will facilitate the evaluation and analysis of safety information from all gene transfer clinical trials. Reports from the database will inform diverse user groups such as IRBs, IBCs, local DSMBs, investigators, research participants, and the general public. Currently, the basic data structure and software design are nearing completion, and a draft common adverse event (AE) reporting form acceptable to both NIH and FDA staffs has been completed. The next steps include obtaining input from other user groups to finalize system software and training investigators and sponsors in the use of controlled vocabularies.

B. Mr. Shipp

Mr. Shipp summarized the public comments on the Proposed Action for SAER. Thirty-four sets of comments were received: two from professional associations, one from a scientific association, three from industry associations, six from patient groups and associations, three from academic officials, four from pharmaceutical and biotechnology companies, and the remainder from individuals. According to those comments, the prohibition of submission of individually identifiable patient data was supported

universally. Public access was also generally favored although there were differing views about the definition of confidential commercial information. Regarding the timing and scope of SAER, the majority of comments favored harmonization; however, industry and the National Hemophilia Foundation believe that no raw SAEs should be reported to the RAC, but rather that the RAC should rely on FDA for that information. A majority of respondents stated their belief that the RAC and the proposed GTSAB can serve a unique and necessary role in the public dissemination of safety and ethical information regarding gene transfer research (GTR) given that FDA is bound by confidentiality restrictions.

C. **Public Comment**

1. **Abbey S. Meyers, National Organization for Rare Disorders (former RAC member)**

Ms. Meyers described one role of the RAC as informing a public fearful of gene transfer research. In the wake of the Jesse Gelsinger tragedy and problems with genetically modified foods, public trust is eroding. The proposal is necessary to prevent the rejection of gene therapy as is happening with agricultural biotechnology. Gene therapy will fail if the public withdraws its trust in research, the researchers and the government's ability to protect the people. She urged the adoption of the proposed action and suggested that industry needs to realize that gene therapy is not just about money; it is about lives.

2. **Stephan E. Lawton, Biotechnology Industry Organization (BIO)**

Mr. Lawton began with assurances that BIO supports the reporting and analysis of safety data. However, they interpret the proposed action as, for the first time in the history of DHHS, compelling the submission and revelation of confidential commercial information to the public. This would make information accessible to competitors and could constitute a significant risk to smaller biotechnology companies, particularly in their ability to attract venture capital. He requested an invitation to work with the RAC/NIH on the proposed action prior to its approval.

Dr. Mickelson requested clarification of BIO's position in light of the fact that this same type of information has been requested, released, and discussed by the RAC for a decade. Mr. Lawton replied that some of the information requested in Appendix M could be of advantage to competitors; therefore, they objected to not being able to label it trade secret. Dr. Patterson reiterated that the proposal refers to a set of data that has already been requested for 10 years with the provision that it not include confidential commercial information. If it is marked as such, decisions will be made on a case-by-case basis allowing for dialogue with the investigator. She emphasized the need to be true to the spirit of the proposal to which Mr. Lawton requested again to work with NIH on the letter of the proposal.

3. **Rosemary Quigley, Council of Public Representatives (COPR)**

By speakerphone, Ms. Quigley expressed her concerns about the adequacy of research subject protection and the need for patient access to information necessary for truly informed consent. COPR strongly supports adoption of the proposed action as drafted. In order to protect participants and advance the nascent field of GTR, she stressed the importance of reporting all adverse events when there is any possibility of association with the gene transfer product. The creation of the GTSAB was endorsed as a necessary complement to the reported raw data that may become available under the FDA proposal. She stated her appreciation that in addition to the RAC review of protocols, NIH would now take the responsibility for the informed dissemination of SAE information. Regarding the BIO statement, COPR views public disclosure of SAEs and discussion of the analyzed data as assistance, not a hindrance, to industry.

4. **Paul Gelsinger, Citizen**

Mr. Gelsinger stated his belief that a major reason for his son's death in a gene transfer clinical trial was the financial pressure upon medical research that caused money to become more important than the welfare of clinical trial participants. He urged researchers to properly report all AEs and to allow NIH to

discuss and review events related to GTR, and that FDA be allowed to release more information to the public. He stated that this proposed action is an appropriate step toward getting GTR on the correct path.

5. **W. French Anderson, University of Southern California/American Society of Gene Therapy (ASGT)**

Speaking on behalf of ASGT, Dr. Anderson stated that ASGT is very much in favor of the Proposed Action and the proposal to allow FDA to be more open regarding SAE reports. Although he expressed support for the spirit of these proposals, he was concerned that, in the enthusiasm to implement them, certain aspects could cause problems, so he suggested working with BIO and other individuals.

6. **Alan Milstein, Attorney**

Mr. Milstein queried the RAC about the meaning of Mr. Lawton's statement that "we can work out" the concerns of the biotechnology industry. He was apprehensive that negotiations might result in the removal of the requirement for public disclosure of SAEs.

D. RAC Discussion

Issues discussed included the following:

- Dr. Skirboll clarified the following points: SAEs would be submitted to NIH in a manner harmonized with FDA submission. The GTSAB analysis would not occur in public, but the reports generated would be publically discussed by the RAC. As with any raw data that come to NIH, this data would also be publicly accessible if requested under the Freedom of Information Act (FOIA). Should there be any substantive changes to the proposal, it would have to be brought back to the RAC for another vote. Because the *NIH Guidelines* can be amended if necessary, further changes may be made should the FDA public disclosure rule become regulation.

- Ms. King reminded everyone present about the language in Appendix M of the *NIH Guidelines* about proposals not containing trade secrets or confidential commercial information; she reiterated that nothing in the Proposed Action changes that language, which has been in effect for about 10 years, and she suggested that RAC discussion center on aspects of the Proposed Action other than the wording found in Appendix M.

- Dr. Markert stated that GTR is not particularly high risk in relation to other research; however, the Proposed Action is necessary to allay the public perception of it as such. Another misconception is that the GTSAB would be reviewing individual SAEs. In actuality, it would review data in the aggregate. Individual review of SAEs could continue to be the responsibility of the local DSMBs. Dr. Markert also noted that a database of raw SAE information on the Web that can be accessed by anyone may be a disservice to the public. Dr. Patterson and Dr. Greenblatt explained that while analysis of the data would be available, the raw data and the preanalysis would be sheltered behind a firewall. Raw data would be available only through FOIA requests to OBA.

- Dr. Jay P. Siegel, FDA, explained that FDA does assess AE in a manner similar to that proposed for the GTSAB, but FDA recognizes that this potential duplication of effort is currently necessary due to the restrictions on public disclosure by FDA. In the event that FDA's disclosure rules are loosened, it would be appropriate to review the coordination of FDA and NIH efforts. Dr. Siegel described some of the issues related to the review of safety data, particularly noting that the aggregate assessment of safety data is a complex process. He further noted that the GTSAB will likely review a database that is somewhat different from the one FDA reviews because of disclosure issues. Dr. Siegel reiterated FDA's position that periodic overview of SAE data in the public domain is a positive development, and that FDA will work with the GTSAB and will continue to work with OBA and the RAC.

- Dr. Aguilar-Cordova brought up the suggestion by ASGT and others that SAEs be reported in their clinical context. He suggested a possible role for the GTSAB in organizing Gene Transfer Policy

Conferences (GTPCs), and properly disseminating the information put forth at these conferences to the public, investigators, and sponsors.

- Dr. Gordon stated that creation and utilization of the GTSAB and AE database are essential. A usable database in the hands of experts can bring forth important trends in GTR that may prevent an SAE and identify potentially promising areas.

- Dr. Breakefield agreed with Dr. Aguilar-Cordova's comments about the necessity of having mechanisms in place so that knowledgeable people from different sectors of GTR can meet quickly and efficiently, analyze SAE data, and release the analysis publicly. She explained that the existence of more safety nets means a better chance of detecting a potential problem before it becomes serious.

- Dr. Friedmann commented that while the proposal may be imperfect, it does address many of the important issues in the GTR field. He advocated approving the proposal as it is, implementing it, and then being flexible in dealing with problems as they arise. He stressed the importance of the interaction among Government, academia, and industry as being necessary to move the gene transfer field forward.

- Dr. Macklin reminded RAC members that policies rarely include operational details; fine-tuning those details occurs during implementation. She also stated that overlapping responsibilities are not necessarily negative if they result in improved protection of human subjects participating in frontier research areas such as GTR.

- Dr. Greenblatt declared his strong support for the creation of the GTSAB, stating that it would represent a significant improvement over what is currently available and that it has value to patient-subjects and to science. He pointed out that the GTSAB would be reevaluated after two years.

E. Committee Motion 4

It was moved by Dr. Gordon and seconded by Ms. Levi-Pearl that the RAC recommend the Proposed Action to amend the *NIH Guidelines* to the NIH Director with the understanding that the details will be worked out. The vote was 11 in favor, 0 opposed, and 1 abstention.

VII. Discussion of Human Gene Transfer Protocol #0101-445: Clinical Protocol for Wild-Type p53 Gene Induction in Premalignancies of Squamous Epithelium of the Oral Cavity via an Adenoviral Vector

Principal Investigator:	Gary Clayman, M.D., University of Texas M.D. Anderson Cancer Center
Sponsor:	Introgen Therapeutics, Inc., represented by Deborah R. Wilson, Ph.D.
RAC Reviewers:	Drs. Aguilar-Cordova, Breakefield, Chow, and Macklin
Ad Hoc Reviewer:	Carter Van Waes, M.D., Ph.D., National Institute on Deafness and Other Communication Disorders, NIH

A. Protocol Summary

For a discrete group of patients with preneoplastic lesions of the oral cavity, no meaningful treatment exists other than conventional surgery. Surgery does not address the multifocality, high incidence of recurrence, and second primary lesions involving aerodigestive tract sites. Biochemoprevention approaches have demonstrated disappointing results; in more than 50% of patients, lesions become malignant. Biomarker studies have suggested that patients with mutant p53 and genetic instability were at greatest risk of disease progression. The objective of this protocol is to directly modify the precancerous cell to express large quantities of an exogenously introduced, normal tumor suppressor gene product that

may reverse the premalignant process by inducing apoptosis in the cancer predisposed cells, allowing for repopulation with normal genotype epithelial cells. The goal is to determine the transduction efficiency of adenoviral mediated wild-type p53 gene transfer in reversing oral premalignancies.

Patients will receive an injection of the Ad5CMVp53 vector and oral rinse on day 1 followed by twice-daily oral rinses on days 2-5, additional lab work, research blood draws and photo documentation for the completion of one cycle. The study cycle will be repeated on a monthly basis for a period of 6 months. A total of 12 patients will be entered into the phase I dose escalation study with 33 patients anticipated to be entered into the phase II study. Biopsies of normal and preneoplastic tissue are performed at pretreatment and two hours following the first oral rinse of the 1st and 6th cycles. Alternative biologic endpoints will also be monitored through the collection of serum and urine. Maximal transduction rate will be determined by immunohistochemistry of p53 and downstream gene products.

B. **Written Comments From Preliminary Review**

Three RAC members recommended that the protocol warranted public discussion. Drs. Breakefield, Chow, and Macklin submitted written reviews, as did *ad hoc* reviewer Dr. Van Waes, to which the investigators responded in writing and during this meeting.

Dr. Aguilar-Cordova raised no safety concerns. He noted that adenoviruses are relatively unstable at low pH and queried the investigators about the effect of saliva on the adenovirus.

Dr. Breakefield focused on the novel route of administration (oral rinse), which is difficult to model in animals and may have toxic consequences to organs such as the larynx. Because premalignancies were targeted, she was also concerned about the risk-benefit ratio since it was not clear how well the vector would be able to transduce the target cells by this route and, if it did, whether the transduced cells would undergo apoptosis. Given that smoking and alcohol consumption predispose squamous cell carcinomas of the oral cavity, she asked whether participants would be counseled about these risks. Dr. Breakefield also inquired about the stability of the adenoviral vector in saliva, how the saliva will be monitored for shed virus after vector administration, and how SAEs associated with the oral tissues and larynx would be monitored.

Dr. Chow also focused on the route of administration and the targeted disease. She expressed concern about the possible effects of the oral rinse and the 10 percent acetic acid prerinse on nontarget tissues in the oral cavity as well as possible accidental exposure to the epithelial cells lining the airway and the esophagus. Since a control arm using a placebo oral rinse is not proposed, Dr. Chow wondered how investigators would know whether any observed effect was due to the intralesional injection of the virus, the oral rinse, or both.

Dr. Macklin focused on recruitment of participants, how and where it would occur and who would be doing it. She also expressed concerns about the route of administration and the inability to model it in animals prior to human trials. She questioned whether compliance with a 30-minute oral rinsing regimen would be possible, and pointed out that possible harm could result from swallowing or aspirating the virus solution. Overall she expressed concern about the risk-benefit balance, suggesting that the uncertainty of potential benefits may not outweigh the potential harms. In the informed consent document, the terms "patient," "treatment," and "doctor" should be replaced with terms that reflect the experimental nature of the process.

Dr. Van Waes also centered on the use of a new patient population and delivery method. He asked for the percentage of dysplasias that have p53 mutations, the frequency with which lesions with p53 mutations progress to carcinoma, why p53 mutation is not an eligibility requirement, and whether preclinical studies have been performed to support the hypothesis that wt-p53 can efficiently induce apoptosis of premalignant cells and repopulation of normal epithelial cells. He also asked about the rationale and safety of the oral acetic acid rinse, whether acetic acid is a carcinogenic agent in subjects using tobacco and alcohol, and why intralesional injection without the rinse is not being performed first. Dr. Van Waes also suggested that the consent document include a description of the rinse and instructions for research subjects to abstain from oral contact with others.

C. RAC Discussion

Ms. Levi-Pearl requested that the informed consent document include financial disclosure information.

Dr. Macklin commented on the "therapeutic misconception" and the need for a clearer distinction in the protocol between the role of researcher and that of a personal physician.

Dr. Van Waes requested that the investigators amend the eligibility criteria to make it clear that they are recruiting participants who have failed other therapies and who have widespread or diffuse disease involvement.

Dr. Friedmann asked the researchers to explain why leukoplakia is not part of the study, and to describe the fate of all the administered adenovirus, particularly whether it survives in the trachea.

D. Investigator Response

Dr. Clayman clarified that the protocol is directed toward participants who have failed other standard or experimental treatments. Fifty percent of patients diagnosed with premalignancy progress to the malignancy within 6 months.

In regard to the delivery route, preclinical studies showed no toxicity in mice receiving an equivalent oral dose. Also there have been other trials involving intratumoral injection in which participants have been found to shed the same vector in saliva without ill effect. A 30 minute oral rinse is standard in other treatments for head and neck squamous cell carcinoma patients. The use of the 10 percent acetic acid did not significantly change the pH of the oral cavity, and ingested adenovirus p53 is neutralized by the stomach's pH of 1.

Dr. Clayman explained that leukoplakias are not necessarily premalignant. They can be benign long-term processes that do not progress to malignancy.

E. Public Comments

No public comments were offered.

F. RAC Recommendations

Dr. Mickelson summarized the following RAC recommendations as follows:

- To revise the eligibility criteria to ensure that only patients with diffuse and refractory premalignancies are enrolled.
- With regard to the informed consent document:
    - To include a financial disclosure for the investigator and any sub-investigators (and if any financial conflict of interests, to give details);
    - To replace the word "patients" with "subjects" or "research participants" since this is clinical research rather than provisional medical care; and
    - To revise the informed consent document to reflect the changes agreed to during the preliminary review (e.g. 30 minute oral rinses would occur in a clinical setting where biohazard containers are available).

G. Committee Motion 5

As moved by Dr. Breakefield, the RAC vote on the recommendations was 9 in favor, 0 opposed, and 2 abstentions.

VIII. Proposed Plan for Addressing Issues Related to Institutional Biosafety Committees/ Allan Shipp, M.H.A., Office of Biotechnology Activities; Cynthia Dunn, M.D., University of Rochester Medical Center; and Andrew George Braun, D.Sc., Harvard Medical School

The issues for discussion were as follows: (1) Should the *NIH Guidelines* be amended to clarify when an institution conducting recombinant DNA research may use an offsite IBC, defined as an IBC at another institution or a commercial IBC? and (2) Pending such an amendment, should an interim policy be put into place to promote clarity and consistency in the interpretation of the current *NIH Guidelines*?

OBA proposed to hold a conference in fall 2001 on a range of issues pertinent to IBC function. Conference participants will discuss such matters as the origin of IBCs, the meaning and necessity of local review, the importance of community consultation, the role of IBCs relative to IRBs, the relationship of IBCs to Federal agencies, and specific questions directly germane to the offsite IBC question. By opening a dialog on these matters, the conference will inform the development of any necessary amendments to the *NIH Guidelines*.

A. Mr. Shipp

Mr. Shipp presented an overview of the membership, procedures, and functions of IBCs as defined in the *NIH Guidelines*. The need to review the current policy has been prompted by two types of queries to OBA. Researchers from institutions that do not have adequate resources to set up their own IBCs would like to use IBCs from neighboring institutions. Investigators who are conducting multisite trials have requested the use of commercial IBCs to coordinate review of the research across sites. A policy interpretation is needed that will optimize subject and community protections and research advancement.

A strawman proposal that included two scenarios was put forth for RAC approval. In scenario A, if an institution or its clinical site conducting GTR were to receive NIH support for recombinant DNA research, it would have to set up a local, institutionally accountable, fully compliant IBC. In scenario B, if an institution or its clinical site did not receive NIH support for recombinant DNA research, but the sponsor of the research did receive NIH support, the site would have to set up its own local, compliant IBC or hire an offsite IBC by contract, with OBA approval. Alternatively, the sponsor IBC could conduct the review, or the sponsor could hire an IBC by contract, with OBA approval. To be acceptable, an offsite IBC used under contract would have to meet the fundamental requirements specified in the *NIH Guidelines* including:

- A majority of the members (three or more) must fulfill the expertise requirements specified in the *NIH Guidelines* (but the expert members need not reside at or be affiliated with the site).
- At least two members must be from the community surrounding the IBC and represent its interests with respect to health and protection of the environment, and these members must be able to consult promptly with other IBC members.
- There must be periodic inspections of the site by the IBC members who have expertise in the type of research being conducted.
- The IBC must be able to be convened as promptly as necessary (which may be done by teleconference).

OBA's ongoing concerns about offsite IBCs included those related to research that occurs in "doc-in-the-box" settings (e.g., in a doctor's office), managing the risks of certain classes of vectors, adequate training of personnel, and ensuring institutional accountability.

B. Dr. Dunn

Dr. Dunn described offsite or independent IBCs as only overseeing GTR clincal trials at biosafety levels 1 or 2. Members must have the required expertise but need not be affiliated with the site. Membership will include a biosafety officer, and infection control specialist from the local community to inspect the site. She cited the trend in which clinical research is shifting from academic medical centers to smaller sites

that may not have the professional expertise to support their own IBCs. Independent IBCs could combine the benefits of local review—community awareness and familiarity with the research environment—with that of central coordination—greater access to expertise, and decrease in conflicts of interest because there would be no direct connection to the clinical site. Dr. Dunn urged OBA to issue a clarification statement indicating that compliance with the *NIH Guidelines* regarding IBCs is not dependent on whether the IBC is constituted internally or independently.

C. Dr. Braun

Dr. Braun noted that he was speaking for himself, not as a representative of Harvard University.

IBCs were originally established so local communities could become more aware of research in their neighborhoods; therefore, meetings should continue to be open to the public, whether the IBC is internally or externally constituted. At most institutions, serving on an IBC is a difficult job that is rarely rewarded properly. Members are motivated by the interesting work, and knowing that they are working for the good of their institution, their field, and their own consciences. It is unclear whether commercial IBCs could be expected to display the same degree of devotion to their work.

However, some aspects of outside IBCs would be useful: highly specialized knowledge could be made available to small institutions, economies of scale would occur when people work full time on one issue, the potential for conflict of interest among academic colleagues would decrease, and improved cooperation among different sites in the same protocol may occur if a single IBC oversaw the biosafety issues at those multiple sites.

A possible drawback to commercial IBCs would be the creation of a situation in which members have greater loyalty to their employer than to the sponsor, the institution at which the research is being conducted, or the research participants. Also if clinical studies were removed from local IBC responsibility, service on the local IBC would be less interesting, resulting in more difficultly in getting volunteers to serve on the local IBCs.

Dr. Braun summarized his view that outside IBCs can provide a useful role in certain circumstances related to the need to provide expertise in human gene tranfer protocols for small clinical establishments. Because there is no substitute for local knowledge or experience, the RAC should strongly encourage clinical sites to establish their own IBCs.

D. RAC Discussion

Dr. Friedmann asked for basic information about the Western Institutional Review Board (WIRB). Dr. Dunn responded that the WIRB is an independent company that was established in 1968 to conduct IRB reviews. WIRB members are paid honoraria by either the clinical site or the sponsor on a per-review basis, whether or not the study is approved.

Dr. Juengst pointed out that the definition of "community member" as a local biosafety officer and an infectious disease expert differs from the type of community member added to an academic IBC: a lay person representing the perspective of the surrounding community. Dr. Dunn responded that the community members are familiar with community attitudes, but they are not necessarily lay members. Dr. Mickelson reiterated the concern that the community-member representation should include lay persons from the public.

Dr. Breakefield suggested the possible establishment of regional IBCs to which institutions would contribute expertise. Another important topic for the proposed fall 2001 meeting, for both independent and institutional IBCs, would be a method of public notification of IBC meetings.

Dr. Aguilar-Cordova suggested that the discussion also include how IBCs function for an institutionally affiliated (but geographically distant) research site, especially in light of how community members are involved in the IBC process.

Dr. Patterson asked the RAC for guidance about whether OBA should adopt the proposed strawman

interim policy, adhere to a strict interpretation of the *NIH Guidelines* on this topic, or make decisions on an *ad hoc* basis until the conference. She also asked whether decisions should take into account the level of risk involved.

Dr. Friedmann preferred to postpone major decisions until more information could be learned during the policy conference. However, Dr. Dunn noted that, if the RAC does not make a decision about the use of independent IBCs before the fall of 2001, sponsors seeking to establish IBC review would be prohibited from using investigative sites outside of academic institutions.

Dr. Macklin suggested that the RAC reject a narrow interpretation of IBCs as being "at the clinical site" in favor of IBCs that provide the most expertise. Dr. Breakefield stated that certain protocols would lend themselves more easily—and with more "comfort" within the community—than the use of independent IBCs. Dr. Aguilar-Cordova agreed that not having a strict interpretation of "at the site" for IBCs would be an acceptable interim stance so that OBA could make case-by-case analyses until after the fall 2001 IBC meeting.

Dr. Braun and Dr. Mickelson objected to the statement in the strawman proposal that the meetings of an independent IBC be allowed to be held by teleconference because teleconferencing would defeat the purpose of allowing public participation and involvement.

E. Public Comment

Dr. J. Tyler Martin, representing Valentis, suggested the need for a "scenario C" to cover sites and sponsors that voluntarily submit to RAC review.

F. Vote of the Committee

As moved by Ms. Levi-Pearl and seconded by Dr. Aguilar-Cordova, the RAC accepted the outline of the strawman proposal until such time as the proposed IBC conference is held with a friendly amendment regarding teleconferencing. The vote was 7 in favor, 3 opposed, and 0 abstentions.

IX. Data Management/Dr. Greenblatt

Dr. Greenblatt reported that 24 new protocols were submitted to OBA during the December 1 to March 1 reporting period; 22 were exempted from public review by the RAC. Of the 449 total protocols, 38 are classified as gene marking, 409 as gene transfer, and 2 as nontherapeutic in normal volunteers. A breakdown of the 409 GTR protocols indicates that:

- 280 were for cancer.
- 50 were for monogenic diseases (cystic fibrosis was the most frequent).
- 35 were for infectious diseases (all but 1 for HIV).
- 44 were for other diseases (coronary artery disease and peripheral artery disease being the most frequent).

A. Amendments and Updates and Adverse Events

During the reporting period, 37 amendments and updates were submitted to OBA including annual updates, eligibility criteria updates, and site additions. Three responses to Appendix M-I-C-1 following the initiation of the clinical investigation were also received.

Of the 206 serious or unexpected reports submitted to OBA, 160 were initial reports and 46 were followups; 25 percent of these occurred prior to 2001. Of the 38 reports classified as serious, possibly

associated, and unexpected, 22 were initial reports and 16 were followups.

Dr. Greenblatt described one report in which a research participant received adenoviral p53 gene transfer for ovarian cancer and died a week after receiving the vector. The preliminary autopsy noted severe peritonitis which was possibly related to treatment. However, the final autopsy attributed death to the complications of extensive metastatic carcinoma, changing the AE from possibly related to unrelated.

X. Food and Drug Administration's Proposed Disclosure Rule/Dr. Noguchi

Dr. Noguchi described FDA's proposed disclosure rule, "Availability for Public Disclosure and Submission to FDA for Public Disclosure of Certain Data and Information Related to Human Gene Therapy or Xenotransplantation," which was published for comment in the January 18, 2001 *Federal Register*. The purpose of the rule is to allow FDA to participate fully in public discussions about GTR and xenotransplantation. While the proposed rule would maintain the confidentiality of information about research participants, trade secrets, and confidential commercial information, it proposes to disclose:

- Product and participant safety data and related information;
- Name and address of the sponsor;
- Clinical indications to be studied;
- A protocol for each planned study, including abstracts, statement of objectives, names and addresses of investigators, names and addresses of official contacts for local review bodies, criteria for subject selection and exclusion, and description of the treatment that will be administered to subjects, as well as the clinical procedures, laboratory tests, or other measures to monitor safety and minimize risk;
- Written informed consent documents;
- Identification of the biological product and method of production;
- Investigational new drug (IND) safety reports;
- Information submitted in the annual report;
- Regulatory status of the IND; and
- Other relevant data and information.

A. RAC Comments

Dr. Greenblatt asked Dr. Noguchi how this information would be made available to the public. Dr. Noguchi responded that the sponsor will submit redacted information with each official submission to FDA. The redacted information will then be sent to FDA's public dockets, which are publicly available on the Internet and updated daily. Dr. Greenblatt expressed concern that the proposal, if implemented, will make all raw SAE data available, which the RAC has previously stated may not be in the public interest. Considering that this rule would be a major departure from past FDA policy, he asked whether Congress would allow it to take effect. While acknowledging the possibility of Congressional opposition, Dr. Noguchi indicated that the proposed rule is consistent with law enacted in 1902 that ensures public confidence in medical therapies involving biological products.

Dr. Friedmann, Ms. King, and Ms. Levi-Pearl commended FDA for taking this significant step toward greater transparency. Dr. Aguilar-Cordova queried how this proposal would relate to the OBA-proposed database. Dr. Noguchi responded that the proposal is intended to augment the OBA database, and the information released publicly by FDA would be available for inclusion in the OBA database.

Ms. Levi-Pearl urged anyone with an opinion about the proposal to provide public comment during the

comment period. Dr. Noguchi also encouraged comments and noted that the deadline is mid-April 2001.

B. Public Comments

1. Dr. Andrew Braun, Harvard Medical School

Dr. Braun suggested that the raw data for SAEs need a denominator—the total number of people studied so that the number of SAEs can be put into context. If this background is not provided, reported numbers may be misleading.

2. Jo Ann Blake, Citizen

Ms. Blake asked whether SAE data such as that described by Dr. Greenblatt will link directly back to the original document in FDA records. If the proposed rule changes are implemented, Dr. Noguchi responded that this would be possible.

C. Committee Motion 6

As moved by Dr. Breakefield and seconded by Ms. King, the RAC voted unanimously (9) to support the implementation of FDA's proposed disclosure rule because it will further the RAC's mandate and is in the public interest.

XI. Chair's Closing Remarks/Dr. Mickelson

Dr. Mickelson thanked the RAC members and indicated that the next RAC meeting is scheduled for June 14-15, 2001.

XII. Adjournment/Dr. Mickelson

Dr. Mickelson adjourned the meeting at 5:25 p.m. on March 8, 2001.

[Note: Actions approved by the RAC are considered recommendations to the NIH Director; therefore, actions are not considered final until approved by the NIH Director.]

Amy P. Patterson, M.D.
Executive Secretary

I hereby acknowledge that, to the best of my knowledge, the foregoing Minutes and Attachments are accurate and complete.

Date:

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Chair

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Jennifer Washburn, writer/journalist
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Patricia D. Williams, TheraSolutions
Carolyn Wilson, FDA
Deborah R. Wilson, Introgen Therapeutics
Gary L. Yingling, McKenna & Cuneo

Attachment III
Abbreviations and Acronyms

AAV	adeno-associated virus
AE	adverse event
AIDS	acquired immunodeficiency syndrome
ASGT	American Society of Gene Therapy
BIO	Biotechnology Industry Organization
CC	Clinical Center, NIH
COPR	Council of Public Representatives
DNA	deoxyribonucleic acid
DSMB	Data and Safety Monitoring Board
E. coli	*Escherichia coli* bacterium
FDA	U.S. Food and Drug Administration
FOIA	Freedom of Information Act
GTPC	Gene Therapy Policy Conference
GTR	gene transfer research
GTSAB	Gene Transfer Safety Assessment Board
HIV-1	human immunodeficiency virus type 1
IBC	Institutional Biosafety Committee
IND	investigational new drug
IRB	Institutional Review Board
LTR	long terminal repeat
MMF	mycophenolate mofetil
NHF	National Hemophilia Foundation
NHLBI	National Heart, Lung and Blood Institute
NIH	National Institutes of Health
NIH Guidelines	*NIH Guidelines for Research Involving Recombinant DNA Molecules*
OBA	Office of Biotechnology Activities
OD	Office of the Director, NIH
PBMC	peripheral blood mononuclear cells
PBSC	peripheral blood stem cells
PCR	polymerase chain reaction
PI	principal investigator
RAC	Recombinant DNA Advisory Committee
RCR	replication competent retrovirus
RG	risk group
RNA	ribonucleic acid
SAE	serious adverse event
SAER	serious adverse event reporting
TBI	total body irradiation
VSVG	Vesicular Stomatitis Virus G
WIRB	Western Institutional Review Board
wt-HIV	wild-type human immunodeficiency virus

EXHIBIT 3

Interactions of HPV E6 and E7 Oncoproteins with Tumour Suppressor Gene Products

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INTRODUCTION

The papillomaviruses are a group of small DNA viruses that induce benign skin lesions, including squamous warts and papillomas. These viruses have been isolated and characterized from a variety of vertebrate species including humans. More than 65 different human papillomavirus types (HPVs) have been described thus far, and a subgroup of about 20 HPVs has been associated with lesions of the anogenital tract (reviewed in DeVilliers, 1989). These HPVs can be further divided into two groups: the "low risk" HPVs, including HPV-6 and HPV-11, which are associated with lesions such as condyloma acuminata which generally remain benign, and the "high risk" HPVs such as HPV-16 and HPV-18, which are associated with lesions that can progress to cancer (zur Hausen and Schneider, 1987). Cervical intraepithelial neoplasia (CIN) is recognized as a precursor to cervical carcinoma, and about 85% of cervical

Cancer Surveys Volume 12: *Tumour Suppressor Genes, the Cell Cycle and Cancer*
 0-87969-369-X/92. $3.00 + .00

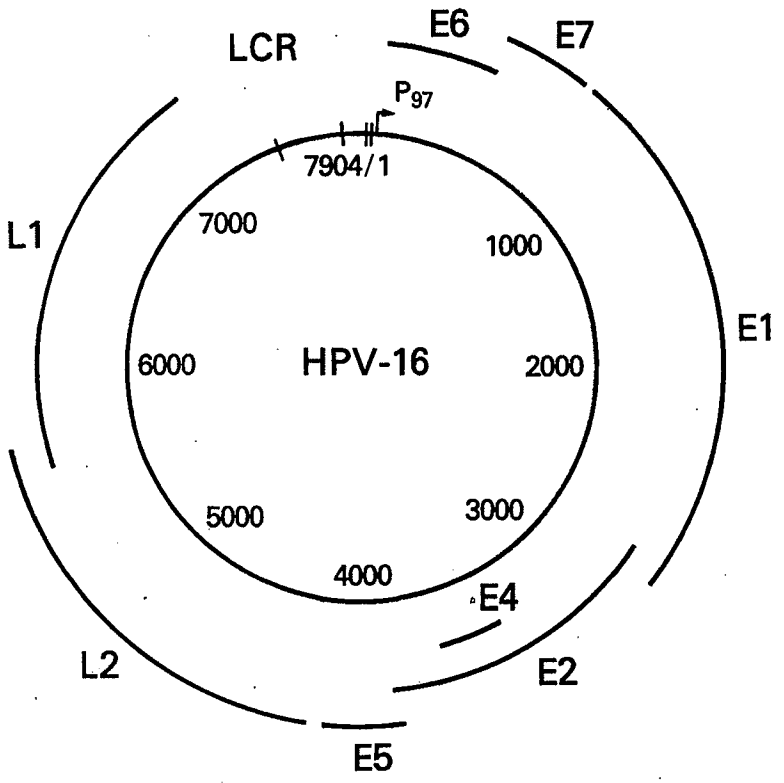

Fig. 1. Circular genomic organization of HPV-16. The early (E) and late (L) open reading frames (ORFs) are represented by the discontinuous outer arcs. The positions of the long control region (LCR) and the early viral promoter P_{97} are also indicated. Vertical marks in the LCR represent the locations of the E2 binding sites ($ACCN_6GGT$) in the genome

carcinomas contain HPV DNA sequences (Riou *et al,* 1990). Further evidence for an active role for HPV in the aetiology of cervical carcinogenesis is derived from studies demonstrating that the "high risk" HPVs can efficiently immortalize human squamous epithelial cells in vitro (Dürst *et al,* 1987b; Pirisi *et al,* 1987; Kaur and McDougall, 1988; Schlegel *et al,* 1988). The viral genomes are double stranded circular DNA molecules of about 8000 base pairs. All of the major open reading frames (ORFs) are located on one strand, and hybridization studies have shown that the viral mRNA species in productively infected cells correspond to that strand of the viral genome. In addition to the coding region, the papillomavirus genomes contain a 1 kbp region with no extensive coding potential. Since this region contains numerous *cis* elements that are important for the control of viral replication and gene expression, it is referred to as the long control region (LCR). A schematic diagram of the HPV-16 genome is shown in Fig. 1. The papillomaviruses have remained refractory to study with standard virological techniques because no tissue culture system that allows their replication has been devised. The failure to grow this virus can be

explained at least partly by the intimate link between papillomavirus gene expression and the differentiation state of the host keratinocyte. Productive functions of the papillomaviruses are expressed only in fully differentiated squamous epithelial cells (reviewed in Baker, 1990). Tissue culture systems for epithelial cells that mimic these differentiation properties have not yet been developed, and many aspects of the papillomavirus life cycle are therefore poorly characterized.

ONCOGENIC FUNCTIONS ENCODED BY HPV

The precancerous CIN lesions contain extrachromosomal HPV sequences (Dürst *et al,* 1985). In the carcinomas, however, the HPV sequences are generally integrated, suggesting that integration may play a part in carcinogenic progression (Boshart *et al,* 1984; Dürst *et al,* 1985; Matsukura *et al,* 1989; Cullen *et al,* 1991). There do not appear to be specific sites of integration of the viral DNA into the host genome (Dürst *et al,* 1987a), but there is a characteristic pattern of integration with respect to the viral genome (Fig. 2). Integration of the viral DNA is probably a random event, but integration patterns that retain expression of the HPV E6 and E7 ORFs and disrupt and/or delete the E1 and E2 ORFs are regularly seen in the cancers, suggesting that they may provide a selective growth advantage to the cell (Schwarz *et al,* 1985; Matsukura *et al,* 1986; Baker *et al,* 1987). Molecular genetic studies have demonstrated that both the E6 and E7 ORFs encode oncoproteins, but the consequences of E1 and/or E2 disruption are less well understood. The E2 ORF encodes a DNA binding protein that interacts directly with its cognate binding sites located within the viral genome to regulate transcription from viral promoters (reviewed in McBride *et al,* 1989). The HPV-16 and HPV-18 E2 gene products have been shown to either activate or repress transcription, depending on the context of the E2 binding sites in the promoter (Phelps and Howley, 1987; Thierry and Yaniv, 1987; Bernard *et al,* 1989; Romanczuk *et al,* 1990; Thierry and Howley, 1991). In HPV-16 and HPV-18, the viral promoter that regulates E6 and E7 expression is repressed by E2. The functions of the HPV E1 ORF have not yet been extensively studied, but by analogy with bovine papillomavirus (BPV) E1, the encoded proteins are likely to be involved in viral replication (reviewed in Lambert, 1991). Recent studies have shown that disruption of either the E1 or the E2 ORF in the context of the full length cloned HPV-16 genome leads to enhanced transformation of primary human foreskin keratinocytes, suggesting that their encoded products directly or indirectly negatively regulate the expression of the viral E6 and E7 transforming functions (Romanczuk H and Howley PM, unpublished).

HPV E7 Oncoprotein

The E7 oncoprotein encoded by HPV-16 and HPV-18 is sufficient for transformation of established rodent fibroblast cell lines such as NIH3T3 cells

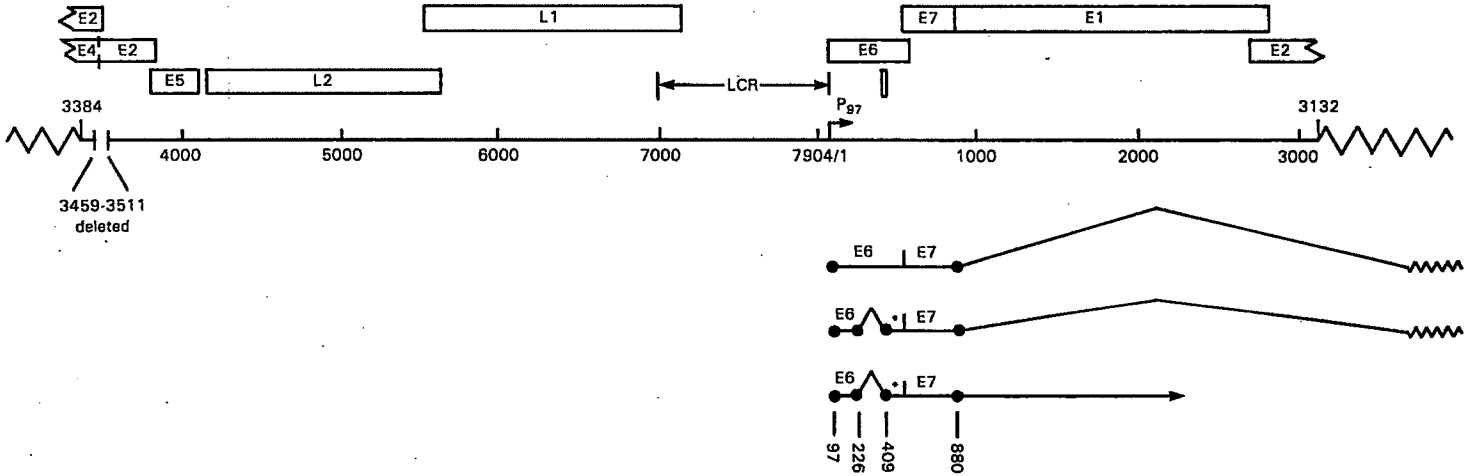

Fig. 2. Structure and expression of the single integrated copy of HPV-16 in the SiHa human cervical carcinoma cell line (Baker *et al*, 1987). Integration led to a disruption of the E2 ORF, and bases 3132 to 3384 and 3459 to 3511 have been deleted. The major viral mRNA species detected are expressed from the P_{97} promoter and are shown below. They all encode the full length E7 protein as well as the full length or internally spliced versions of E6, designated E6* (from Smotkin and Wettstein, 1986; Baker *et al*, 1987)

(Kanda *et al,* 1988; Phelps *et al,* 1988a; Vousden *et al,* 1988; Watanabe and Yoshiike, 1988; Bedell *et al,* 1989; Tanaka *et al,* 1989). Expression of the E7 gene induces focus formation in these cell lines and results in anchorage independence and tumorigenicity in nude mice. E7 has properties similar to adenovirus (Ad) E1A in that it can *trans*-activate the Ad E2 promoter and can cooperate with the activated *ras* oncogene to transform primary baby rat kidney cells (Matlashewski *et al,* 1987; Phelps *et al,* 1988a,b; Storey *et al,* 1988). Biochemical studies have revealed that HPV-16 E7 encodes a nuclear, zinc binding 98 aminoacid phosphoprotein (Smotkin and Wettstein, 1986; Sato *et al,* 1989a). The aminoterminal 38 aminoacids are strikingly similar to portions of conserved regions 1 and 2 of the Ad E1A proteins, as well as to the analogous regions of SV40 T antigen (Fig. 3) (Phelps *et al,* 1988a,b). Genetic studies of Ad E1A, SV40 T antigen and HPV E7 have shown that the conserved regions are required for cellular transformation (Kalderon and Smith, 1984; Lillie *et al,* 1987; Moran and Mathews, 1987; Cherington *et al,* 1988; Whyte *et al,* 1988a; Larose *et al,* 1991), as well as for the interaction with a number of host cellular proteins including the retinoblastoma tumour suppressor gene product pRB (DeCaprio *et al,* 1988; Münger *et al,* 1989a; Whyte *et al,* 1989). At the carboxyterminal boundary of the conserved aminoacid sequences are two serine residues, which can serve in vitro as substrates for phosphorylation by casein kinase (CK) II (Firzlaff *et al,* 1989; Barbosa *et al,* 1990). The carboxyterminal portion of E7 does not share any additional sequence similarity with Ad E1A or SV40 T antigen. It contains two copies of a Cys-X-X-Cys sequence motif, which are likely to be involved in the zinc binding property of E7 (Barbosa *et al,* 1989). The major properties of E7 are summarized in Table 1.

HPV E6 Oncoprotein

There are several species of viral mRNAs in cervical carcinomas and derived cell lines (Schwarz *et al,* 1985; Smotkin and Wettstein, 1986; Baker *et al,* 1987). One species has the capacity to encode the full length E6 protein, and several spliced versions of E6 (called E6*) are also produced (see Fig. 2). These internally spliced versions of E6 are unique to the "high risk" HPV types, and thus far, no biological activities have been described for the shortened E6* proteins potentially encoded by these spliced mRNAs. It is speculated that the E6* spliced mRNAs would be more efficiently translated into the E7 protein by bringing the initiation codon for E7 closer to the 5′ end of the polycistronic mRNA (Smotkin *et al,* 1989). Moreover, this splicing event could constitute a mechanism for regulating the relative levels of E6 and E7. The full length E6 protein has transforming properties in that it is necessary for efficient transformation of primary human cells of both epithelial and fibroblastic origin (Hawley-Nelson *et al,* 1989; Münger *et al,* 1989b; Watanabe *et al,* 1989). The major properties of HPV-16 E6 are summarized in Table 2. It encodes a 151 aminoacid, basic, zinc binding protein (Androphy *et al,* 1987;

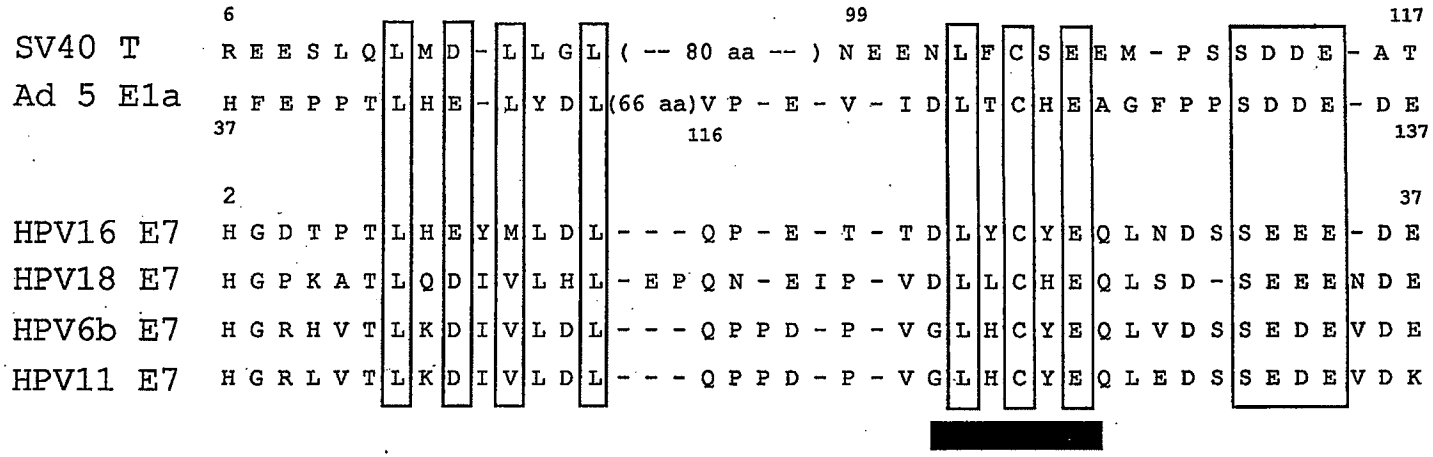

Fig. 3. Aminoacid sequence comparison of regions of SV40 T antigen and portions of conserved regions 1 and 2 of the Ad E1A proteins with the aminoterminal portions of the E7 proteins of the anogenital associated HPVs. The standard one-letter code for aminoacids is used. Identical and isofunctional aminoacid residues are indicated by boxes. The E7 sequences which are necessary for pRB binding (Münger *et al*, 1989a) are indicated by the bar at the bottom of the figure

TABLE 1. Properties of the HPV-16 E7 oncoprotein[a]

Biochemical properties
- 98 aminoacid, zinc binding, acidic nuclear phosphoprotein
- Phosphorylated at serine residue(s) by casein kinase II
- Apparent M_r: 19 kDa; predicted M_r: 11 kDa
- Aminoterminal region (aminoacid 1–39) structurally related to regions of adenovirus E1A and of large T antigens of the polyomaviruses
- Can complex with the retinoblastoma tumour suppressor gene product pRB
- Carboxyterminal region contains two Cys-*X*-*X*-Cys sequence motifs
- Can activate the cellular transcription factor E2F

Biological properties
- Sufficient for transformation of established rodent fibroblasts (eg NIH3T3 cells)
- Adenovirus E1A like transcriptional modulatory and transformation functions:
  - Transactivates adenovirus E2 promoter
  - Cooperates with *ras* to transform primary rodent cells
- Necessary together with E6 for the efficient immortalization of primary human squamous epithelial cells
- Abrogates TGF-β mediated repression of c-*myc* and G_1 growth arrest

[a]See text for references

Barbosa *et al*, 1989; Grossman and Laimins, 1989). It contains four repeats of a Cys-X-X-Cys sequence motif similar to the carboxyterminal region of E7. This led to the suggestion that E6 and E7 are evolutionarily related (Cole and Danos, 1987). Biochemical studies have shown that the E6 proteins derived from the "high risk" HPV types can associate with the tumour suppressor protein p53 (Werness *et al*, 1990). There are no significant aminoacid sequence similarities between HPV E6, Ad E1B and SV40 T antigen, which are all p53 binding proteins. Biochemical studies have indicated that the E6-p53 interaction can lead to the selective degradation of p53 in vitro (Scheffner *et al*, 1990). The E6 protein binds to double stranded DNA with high affinity (Mallon *et al*, 1987; Grossman *et al*, 1989; Imai *et al*, 1989), and there is some evidence that the E6 protein may be involved in transcriptional regulation (Lamberti *et al*, 1990).

TABLE 2. Properties of the HPV-16 E6 oncoprotein[a]

Biochemical properties
- 151 aminoacid, zinc binding, basic protein (pI 9.9)
- Four copies of Cys-*X*-*X*-Cys sequence motif, which may serve as ligands for zinc binding
- Binds to double stranded DNA with high affinity
- Can form a complex with the p53 tumour suppressor protein and promote its in vitro degradation

Biological properties
- Cooperates with E7 for the efficient immortalization of primary human squamous epithelial cells
- May have transcriptional regulatory properties

[a]See text for references

Other Oncogenic Functions Encoded by HPVs

Although the E6/E7 region of the HPV genome is sufficient for cellular transformation in vitro, other HPV genes may also contribute to this phenomenon. In the context of the entire HPV-16 genome, disruption of E1 and E2 leads to increased transformation of primary human foreskin keratinocytes (Romanczuk H and Howley PM, unpublished). Although the exact mechanisms have not yet been delineated, it is presumed that disruption of E2 expression may lead to increased expression of the E6/E7 ORFs, resulting in enhanced transformation. Whether the effect of E1 disruption is mediated through the same transcriptional regulatory pathway is unknown, and other mechanisms involving the direct interaction of E1 and/or E2 with cellular factors cannot be excluded.

The E5 ORF of BPV encodes a small 44 aminoacid, membrane associated oncoprotein (reviewed in DiMaio and Neary, 1990). The BPV E5 protein is associated with a 16 kDa host cellular protein (Goldstein and Schlegel, 1990) and has been implicated in perturbing the growth factor responses of the virally infected host cells (Martin *et al*, 1989; Petti *et al*, 1991). Studies with the "high-risk" HPVs have shown that they also have the capacity to encode E5 proteins (Bubb *et al*, 1988; Halbert and Galloway, 1988). Although they do not share extensive aminoacid sequence homology with BPV-1 E5, they have structural similarities to BPV-1 E5, with a very hydrophobic membrane anchor domain and a more hydrophilic domain. Additional studies are required to define further the biochemical and biological properties of the HPV E5 proteins. Owing to the integration of the HPV genome in the E1/E2 region, expression of E5 is generally not detected in cervical carcinomas and the derived cell lines. However, the possibility that expression of the HPV E5 genes could be important in participating in the HPV associated preneoplastic lesions cannot be excluded.

INTERACTION OF HPV E7 WITH CELLULAR PROTEINS

Interaction of HPV E7 with pRB

It was recognized some time ago that the Ad E1A proteins form specific complexes with several host cellular proteins in vivo (Yee and Branton, 1985; Harlow *et al*, 1986). Although some of these proteins interacted with sequences on the Ad E1A proteins which were recognized to be important for cellular transformation (ie conserved regions 1 and 2 of Ad E1A) (Whyte *et al*, 1988a), the biological importance of these interactions remained unclear. The hypothesis that the transforming functions of Ad E1A could, at least in part, be mediated through some of these protein/protein interactions received considerable support, however, when the 105 kDa Ad E1A associated protein was identified as the product of the retinoblastoma tumour suppressor gene, pRB (Whyte *et al*, 1988b). Given the functional and structural similarity between HPV-16 E7 and Ad E1A (Phelps *et al*, 1988a,b), the possibility of an interaction between the

E7 protein and pRB was investigated and confirmed (Dyson *et al,* 1989a). Coprecipitation experiments provided evidence for the complex in HPV-16 E7 expressing cells (Münger *et al,* 1989b). HPV-16 E7, much like SV40 T antigen (Ludlow *et al,* 1989), preferentially binds the underphosphorylated form of pRB (Münger K, unpublished). Cell cycle studies have shown that pRB has properties of a cell cycle regulatory factor in that its phosphorylation state varies through the cell cycle (Buchkovich *et al,* 1989; DeCaprio *et al,* 1989; Mihara *et al,* 1989). The underphosphorylated forms of pRB are present only during G_0 and G_1 of the cell cycle. Since pRB acts as a negative growth regulator at the G_1/S boundary, these underphosphorylated forms therefore must represent the forms of pRB that confer G_1 growth arrest. According to this model, G_1-S cell cycle progression is achieved by phosphorylation of pRB through the action of cell cycle specific serine/threonine protein kinase. Phosphorylated forms of pRB are present during S, G_2 and early M phases in the cell cycle and are dephosphorylated during M phase. These observations have led to the hypothesis that complex formation of the underphosphorylated forms of pRB with Ad E1A, SV40 T antigen or HPV E7 results, much like phosphorylation, in an "inactivation" of pRB and cell cycle progression (Fig. 4) (reviewed in Green, 1989). Ad E1A, SV40 T antigen and HPV E7 each has mitogenic activity and can induce DNA synthesis in quiescent cells (Mueller *et al,* 1978; Moran and Zerler, 1988; Sato *et al,* 1989b). This property of the DNA tumour viruses is thought to be essential in placing the infected host cells in a replicative state to permit viral DNA replication.

Quantitative mixing experiments with in vitro synthesized E7 proteins derived from "high risk" and "low risk" HPVs have shown that the E7 proteins of the "high risk" HPVs bind to pRB with higher affinity than the E7 proteins of the "low risk" HPVs (Münger *et al,* 1989a). Whereas the pRB binding affinities of HPV-16 and HPV-18 E7 were comparable, the HPV-11 and HPV-6 E7 proteins bound to pRB with significantly decreased affinities.

The pRB binding site on HPV-16 E7 was mapped using a series of mutant E7 proteins. It is confined to a portion of the region of sequence similarity of the E7 protein with the Ad E1A conserved region 2. The CK II phosphorylation site, which is also in this part of the E7 molecule, does not seem to be required for pRB binding (see Fig. 3) (Münger *et al,* 1989a; Barbosa *et al,* 1990; Firzlaff *et al,* 1991). Competition studies with a series of HPV-16 E7 specific synthetic peptides have confirmed the importance of this domain in binding pRB (Jones *et al,* 1990).

Mutant E7 proteins with a decreased affinity for pRB or unable to bind pRB were also transformation defective in NIH3T3 and in baby rat kidney cell *ras* cooperation assays. In contrast, some mutations in the portion of the E7 protein, which is similar to conserved region 1 of Ad E1A, were transformation defective, although they had wild type affinity for pRB in in vitro binding assays. This led to the conclusion that complex formation with pRB may be necessary but not sufficient for cellular transformation (Banks *et al,* 1990; Phelps WC, Münger K, Yee CL and Howley PM, unpublished).

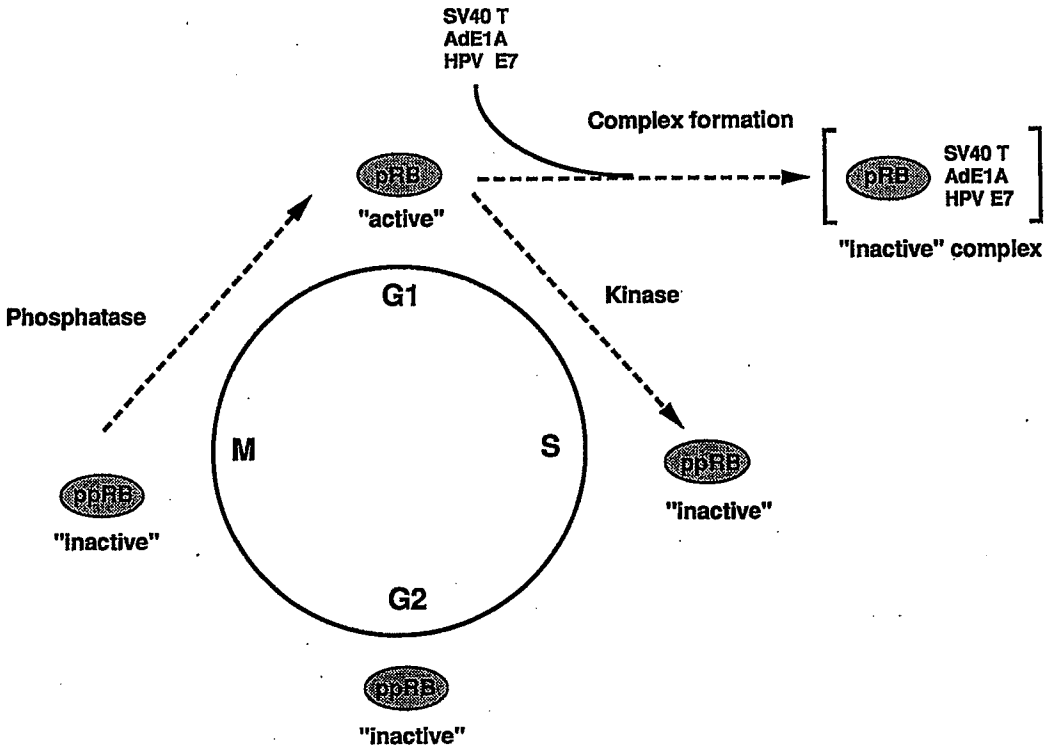

Fig. 4. "Inactivation" of pRB by complex formation with the oncogenes of different DNA tumour viruses. The retinoblastoma tumour suppressor protein is differentially phosphorylated during the cell cycle. The underphosphorylated form (pRB) is only detected during the G_0/G_1 phase of the cell cycle. Since the retinoblastoma protein is thought to act as a negative regulator of cell growth, pRB is regarded as the "active" form. Phosphorylation at serine and threonine residues at the G_1/S boundary is thought to result in an "inactivation" of the retinoblastoma protein and trigger cell cycle progression and entry into S phase. Complex formation of pRB with Ad E1A, SV40 T antigen or HPV E7 may also result in the "inactivation" of the negative growth regulatory functions of pRB and lead to cell cycle progression. Since the regulatory functions of the pRB kinase and phosphatase are annulled by complex formation with the viral oncoproteins, this may result in uncontrolled cell growth and differentiation

Interaction of HPV E7 with Other Host Cellular Proteins

Coprecipitation experiments have shown additional cellular proteins in complex with E7. Some of these E7 associated cellular proteins have electrophoretic mobilities similar to those of Ad E1A and SV40 T antigen associated proteins (Münger K, unpublished). The low levels of E7 present in HPV transformed cell lines have not yet permitted a full characterization of these host cellular proteins. Of particular interest is a protein designated p107, which seems to interact with the identical sequences on Ad E1A and SV40 T antigen as pRB (Dyson *et al*, 1989b; Ewen *et al*, 1989; Whyte *et al*, 1989). In vitro mixing experiments with purified E7 protein or coprecipitation experiments

with E7 specific antibodies led to the detection of a protein with the same electrophoretic mobility as p107. HPV-16 E7 peptides compete with the Ad E1A bound p107. In addition, p107 can be detected in complex with HPV-16 E7 peptides immobilized on Sepharose beads (Dyson N, Münger K, Howley PM and Harlow E, unpublished).

Since mutations in the aminoacid sequence of HPV-16 E7 analogous to conserved region 1 of Ad E1A result in transformation defective proteins despite their ability to bind pRB in vitro with wild type affinity, other cellular proteins that interact with this aminoterminal domain of E7 must also be important for the transformed phenotype. A possible candidate is p300, which interacts with the Ad E1A proteins in conserved region 1 (Whyte *et al,* 1989). To date, there is no direct experimental evidence for an interaction of p300 with the E7 protein.

The E7 proteins encoded by the "high risk" HPVs differ from the "low risk" HPV E7 proteins in their CK II phosphorylation site. The "high risk" HPV E7 proteins are more rapidly phosphorylated by CK II in vitro than the "low risk" HPV E7 proteins (Barbosa *et al,* 1990). Mutations in the E7 phosphorylation site lead to a decrease in transformation without markedly affecting pRB binding (Barbosa *et al,* 1990; Firzlaff *et al,* 1991). The biological consequences of these findings are still unclear.

Ad E2 *Trans*-Activation Function of HPV E7

Since E7 does not share any significant aminoacid sequence similarity with conserved region 3 of Ad E1A, the portion of Ad E1A that has been shown to be important for its strong *trans*-activation function, it was clear that E7 must function differently from the 13S E1A product. The 12S form of Ad E1A, which lacks conserved region 3, is a weak *trans*-activator and functions on a subset of the 13S Ad E1A targets (reviewed in Nevins, 1989). Recent biochemical studies have shown that one of the targeted cellular transcription factors, E2F, is activated by Ad E1A through dissociation of a heteromeric macromolecular complex. This Ad E1A mediated dissociation is dependent on Ad E1A sequences in conserved region 2 and may be mediated by protein/protein interactions (Bagchi *et al,* 1990). Genetic and biochemical studies have shown that HPV E7 *trans*-activates a similar subset of 12S Ad E1A responsive promoters, and it can release E2F from the macromolecular complex. Although the sequences on the E7 protein required for this activity have not been mapped in detail, it is clear that the portion of E7 similar to conserved sequence 2 of Ad E1A is necessary for this activity (Phelps WC *et al,* in press). This is in agreement with previous findings with mutant E7 proteins, which showed that the sequences required for *trans*-activation were not clearly separable from those required for transformation (Edmonds and Vousden 1989; Phelps *et al,* 1990; Watanabe *et al,* 1990). The "low risk" HPV E7 proteins, although impaired for cellular transformation and pRB binding,

can *trans*-activate the Ad E2 promoter with an efficiency similar to that of the "high risk" HPVs (Storey *et al*, 1990; Münger *et al*, 1991). This suggests that cellular transformation and transcriptional *trans*-activation may not be mediated through interactions with an identical set of host cellular proteins.

Abrogation of TGF-β Mediated Repression of c-*myc* Expression

Transforming growth factor-β (TGF-β) acts as a potent negative growth regulator of many epithelial cells and induces G_1 growth arrest (reviewed in Moses *et al*, 1990). TGF-β treatment of keratinocytes results in the rapid transcriptional repression of c-*myc* expression (Coffey *et al*, 1988). Studies with c-*myc* antisense oligodeoxynucleotides have suggested that the observed G_1 growth arrest may be a consequence of this transcriptional repression of c-*myc* expression (Pietenpol *et al*, 1990a). Many squamous epithelial derived tumour cell lines have lost their responsiveness to TGF-β. Moreover, human keratinocyte cell lines transformed by the HPV-16 E6/E7 region expressed from the human β-actin promoter, by HPV-18 or by SV40, are also resistant to growth inhibition by TGF-β (Pietenpol *et al*, 1990b). HPV-16 E7, SV40 T antigen and Ad E1A can each abrogate the TGF-β mediated transcriptional repression of c-*myc* expression in a transient transfection assay. This property is dependent on the integrity of the pRB binding site of each of these viral oncoproteins. This suggests that one of the cellular proteins that can form a complex with these viral oncoproteins through the pRB binding domain such as pRB or p107 may be involved in the transcriptional regulatory pathway of c-*myc* expression (Pietenpol *et al*, 1990b).

INTERACTION OF HPV E6 WITH THE p53 TUMOUR SUPPRESSOR PROTEIN

Like SV40 T antigen and the Ad5 E1B 55 kDa oncoprotein, the E6 proteins encoded by the "high risk" HPVs can form a complex with the tumour suppressor protein p53 (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al*, 1982; Werness *et al*, 1990). For the "low risk" HPV E6 proteins, no complex formation with p53 was detected. The aminoacid sequences of E6 that are necessary for this interaction have not yet been defined, but preliminary studies show that rather than a short linear aminoacid sequence, a specific conformational structure of the E6 protein may be required for the E6/p53 interaction (Huibregtse JM, unpublished). The truncated, internally spliced forms of E6, E6* do not interact with p53 (Werness BA, unpublished). Interaction of SV40 T antigen and Ad5 E1B with p53 leads to an extended half life and increased steady state levels of p53 in SV40 and adenovirus transformed cell lines (Oren *et al*, 1981; Reich *et al*, 1983). The levels of p53 in HPV positive cervical carcinoma cell lines and in HPV transformed keratinocyte cell lines are quite low (Scheffner *et al*, 1991). Indeed, it was previously reported

that HeLa cells that contain HPV-18 contain no detectable p53 protein despite the presence of translatable *p53* mRNA (Matlashewski *et al*, 1986). Biochemical studies have revealed that binding of E6 promotes the degradation of p53 in an in vitro system. The E6 induced degradation of p53 is ATP dependent and involves the ubiquitin dependent proteolysis system (Scheffner *et al*, 1990). This property of E6 may account for the low levels of p53 detected in cervical carcinoma cell lines and in HPV transformed human keratinocyte cell lines.

Cotransfection studies with HPV-16 E7 and wild type and mutant forms of *p53* have shown that mutant *p53* can potentiate the ability of E7 and *ras* to transform baby rat kidney cells and confer growth factor independence on the transformed cell lines. The results with wild type *p53* were somewhat less conclusive. Using an anchorage independence assay in NIH3T3 cells, a decrease in transformation was observed. Whether this was due to a specific transformation inhibiting effect of wild type *p53* or a non-specific toxicity effect of overexpressing wild type *p53* in these cells is unknown (Crook *et al*, 1991a).

IS THE FUNCTIONAL INACTIVATION OF THE RB AND p53 GENE PRODUCTS IMPORTANT IN CERVICAL CARCINOGENESIS?

Although the biochemical nature of the interactions of the viral oncoproteins with p53 and pRB is well documented, the biological consequences of these interactions can still be viewed as speculative. Since the "high risk" HPVs are associated with cervical carcinomas, they provide a unique system to test the hypothesis that the complex formation of the HPV E6 and E7 oncoproteins with the p53 and pRB tumour suppressor proteins may be of significance for cervical carcinogenesis.

Approximately 85% of the human cervical cancers contain HPV sequences (Riou *et al*, 1990), and one can predict that if p53 and pRB are relevant and essential targets of E6 and E7, respectively, then there might be no selective advantage to a tumour for further mutations in their *p53* and *RB* genes, since the functions of the encoded tumour suppressor proteins would presumably be annulled through interactions with HPV E6 and E7 oncoproteins. Furthermore, in the HPV negative cervical carcinomas, one would expect p53 and pRB functions also to be abrogated through a different mechanism, most likely mutation. These predictions were tested on a panel of HPV positive and HPV negative cervical carcinoma cell lines. All the HPV positive cell lines had wild type p53 and normal pRB, whereas both HPV negative cervical carcinoma cell lines contained mutated *p53* and *RB* genes (Crook *et al*, 1991b; Scheffner *et al*, 1991) (Table 3). These experiments not only indicate that the *RB* and *p53* genes are frequently inactivated during cervical carcinogenesis but also strongly suggest that p53 and pRB may be physiologically relevant targets of the HPV E6 and E7 oncoproteins. The interactions of the viral oncoproteins and cellular tumour suppressor proteins detected in coprecipitation assays may

TABLE 3. Status of p53 and pRB in human cervical carcinoma cell lines[a]

Cell line	HPV	p53	pRB
HeLa	HPV-18	wt	wt
C4-II	HPV-18	wt	wt
SiHa	HPV-16	wt	wt
CaSki	HPV-16	wt	wt
Me180	HPV-39 related	wt	wt
C33-A	Negative	Mutated, R_{273} to C	Mutated (exon 20)
HT-3	Negative	Mutated, G_{245} to V	Mutated (exon 13)

wt = wild type
[a]See Scheffner *et al*, 1991, for details

therefore reflect an "inactivation" of some of the important regulatory functions encoded by these tumour suppressor gene products.

Some mutations of the *p53* gene are believed to result in a dominant gain, rather than merely a loss of function. Therefore, it is possible that the E6/p53 interaction may not have the exact same consequences as a p53 mutation but rather result in a "null" phenotype. In this context, it is interesting to note that in one recent study, the HPV negative cervical lesions appear to be associated with a poorer clinical prognosis in that they progress more rapidly and have higher metastatic potential than the HPV positive lesions (Riou *et al*, 1990).

ADDITIONAL FACTORS IN CERVICAL CARCINOGENESIS

Cervical carcinoma eventually develops in only a small percentage of women infected with "high risk" HPVs. This suggests that the infection with a "high risk" HPV constitutes only one step in cervical carcinogenesis. In agreement with this notion is the observation that HPV E6/E7 transformed human keratinocyte cell lines are generally not anchorage independent nor tumorigenic in nude mice (Dürst *et al*, 1987a,b; Pirisi *et al*, 1987; Kaur and McDougall, 1988; Schlegel *et al*, 1988). Progression to a fully transformed tumorigenic phenotype in HPV immortalized keratinocytes was observed upon cotransfection with an activated *ras* oncogene or after continuous passaging of the cell lines for extended periods of time (DiPaolo *et al*, 1989; Dürst *et al*, 1989; Hurlin *et al*, 1991; Pecoraro *et al*, 1991). These observations imply that additional cellular events may also be necessary for the development of cervical carcinoma.

Cytogenetic studies have shown that nine out of nine carcinomas of the uterine cervix had a loss of heterozygosity on the short arm of chromosome 3 (Yokota *et al*, 1989), implicating a possible tumour suppressor gene in that region of the genome. Lesions in the same region of chromosome 3p (3p21) have also been noted in small cell lung carcinoma (Yokota *et al*, 1987; Mori *et al*, 1989).

Additional evidence for host cell mutations in cervical cancers is provided by studies with somatic cell hybrids (Stanbridge *et al*, 1982). The cervical car-

cinoma cell lines HeLa and SiHa when fused with normal human cells reverted to a non-tumorigenic phenotype. Further studies with tumorigenic revertants of such hybrid cell lines or by microcell fusion have genetically mapped this effect to human chromosome 11 (Saxon *et al*, 1986; Koi *et al*, 1989). The exact molecular events and biological consequences of these host chromosomal lesions are not yet clearly understood. It has been proposed that some of these factors may control some aspects of HPV transcription (Rösl *et al*, 1988; Bosch *et al*, 1990; Smits *et al*, 1990; Miyasaka *et al*, 1991). Inactivation of such genes in cervical carcinoma cells would lead to deregulated expression of the HPV E6/E7 genes. In such models, infection with a "high risk" HPV would constitute an important initial event of carcinogenesis. A pool of highly replicative cells would be established, since some of the normal aspects of cell cycle, replication and differentiation control would be abolished by functional inactivation of key cellular regulatory proteins such as p53 and pRB. The expression of HPV E6 and E7 may contribute to chromosomal instability with the accumulation of chromosomal aberrations followed by clonal selection of malignant cells and tumorigenesis. Such models stress the importance of environmental mutagenic cofactors that may contribute to carcinogenic progression. Epidemiological studies have identified a small increase in the relative risk for the development of cervical cancers with cigarette smoking and long term use of oral contraceptives (Vessey, 1986).

SUMMARY

The HPVs associated with anogenital cancers encode two oncoproteins, E6 and E7. Both E6 and E7 can form specific complexes with tumour suppressor gene products. The E7 protein binds to the retinoblastoma tumour suppressor gene product pRB, with a preference for the underphosphorylated, "active" form of pRB. The E7 proteins derived from the "high risk" HPVs bind to pRB with a higher affinity than the E7 proteins from the "low risk" HPVs. The "high risk" HPV E6 proteins can associate with the p53 tumour suppressor protein. This interaction promotes the degradation of p53 in vitro, which presumably accounts for the very low levels of p53 in cervical carcinoma cell lines. The functional inactivation of pRB and p53 by the HPV oncoproteins E7 and E6, respectively, are likely to be important steps in cervical carcinogenesis, since mutations in the *RB* and *p53* genes were detected in HPV negative but not HPV positive cervical carcinoma cell lines. Cytogenetic studies strongly suggest, however, that additional chromosomal changes may be necessary for carcinogenic progression of HPV induced anogenital lesions.

References

Androphy EJ, Hubbert N, Schiller JT and Lowy DR (1987) Identification of the HPV-16 E6 protein from transformed mouse cells and human cervical carcinoma cell lines. *EMBO Journal* 6 989–992

Bagchi S, Raychaudhuri P and Nevins JR (1990) Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. *Cell* **62** 659–669

Baker CC (1990) Bovine papillomavirus type 1 transcription, In: Pfister H (ed). *Papillomaviruses and Human Cancer*, pp 91–112, CRC Press, Boca Raton, Florida

Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA and Howley PM (1987) Structural and translational analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *Journal of Virology* **61** 962–971

Banks L, Edmonds C and Vousden KH (1990) Ability of the HPV16 E7 protein to bind RB and induce DNA synthesis is not sufficient for efficient transformation in NIH3T3 cells. *Oncogene* **5** 1383–1389

Barbosa MS, Lowy DR and Schiller JT (1989) Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. *Journal of Virology* **63** 1404–1407

Barbosa MS, Edmonds C, Fisher C, Schiller JT, Lowy DR and Vousden KH (1990) The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II. *EMBO Journal* **9** 153–160

Bedell MA, Jones KH, Grossman SR and Laimins LA (1989) Identification of human papillomavirus type 18 transforming genes in immortalized and primary cells. *Journal of Virology* **63** 1247–1255

Bernard BA, Bailly C, Lenoir M-C, Darmon M, Thierry F and Yaniv M (1989) The HPV18 E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. *Journal of Virology* **63** 4317–4324

Bosch FX, Schwarz E, Boukamp P, Fusenig NE, Bartsch D and zur Hausen H (1990) Suppression in vivo of human papillomavirus type 18 E6-E7 gene expression in non-tumorigenic HeLa x fibroblast hybrid cells. *Journal of Virology* **64** 4743–4754

Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W and zur Hausen H (1984) A new type of papillomavirus DNA and its prevalence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO Journal* **3** 1151–1157

Bubb V, McCance DJ and Schlegel R (1988) DNA sequence of the HPV-16 E5 ORF and structural conservation of its encoded protein. *Virology* **163** 243–246

Buchkovich K, Duffy LA and Harlow E (1989) The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell* **58** 1097–1105

Cherington V, Brown M, Paucha E, St Louis J, Spiegelmann BM and Roberts TM (1988) Separation of simian virus 40 large-T antigen-transforming and origin binding functions from the ability to block differentiation. *Molecular and Cellular Biology* **8** 1380–1384

Coffey RJ, Bascom CC, Sipes NJ, Graves-Deal R, Weissman BE and Moses HL (1988) Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta. *Molecular and Cellular Biology* **8** 3088–3093

Cole ST and Danos O (1987) Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome: phylogeny of papillomaviruses and repeated structure of the E6 and E7 gene products. *Journal of Molecular Biology* **193** 599–608

Crook T, Fisher C and Vousden KH (1991a) Modulation of immortalizing properties of human papillomavirus type 16 E7 by p53 expression. *Journal of Virology* **65** 505–511

Crook T, Wrede D and Vousden KH (1991b) p53 point mutation in HPV negative human cervical carcinoma cell lines. *Oncogene* **6** 873–875

Cullen A, Reid R, Campion M and Lörincz A (1991) Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasms. *Journal of Virology* **65** 606–612

DeCaprio JA, Ludlow JW, Figge J *et al* (1988) SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54** 275–283

DeCaprio JA, Ludlow JW, Lynch D *et al* (1989) The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell* **58** 1085–1095

DeVilliers EM (1989) Heterogeneity of the human papillomavirus group. *Journal of Virology* **63**

4898–4903

DiMaio D and Neary K (1990) The genetics of bovine papillomavirus type 1 In: Pfister H (ed). *Papillomaviruses and Human Cancer*, pp 113–144, CRC Press, Boca Raton, Florida

DiPaolo JA, Woodworth CD, Popescu NC, Notario V and Doninger J (1989) Induction of human cervical squamous cell carcinoma by sequential transfection with human papillomavirus 16 DNA and viral Harvey ras. *Oncogene* **4** 395–399

Dürst M, Kleinheinz A, Hotz M and Gissmann L (1985) The physical state of human papillomavirus type 16 in benign and malignant genital tumors. *Journal of General Virology* **66** 1515–1522

Dürst M, Croce CM, Gissmann L, Schwarz E, and Huebner K (1987a) Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas. *Proceedings of the National Academy of Sciences of the USA* **80** 3812–3815

Dürst M, Dzarlieva-Petrusevska RT, Boukamp P, Fusenig NE and Gissmann L (1987b) Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene* **1** 251–256

Dürst M, Gallahan D, Jay G and Rhim JS (1989) Glucocorticoid enhanced neoplastic transformation of human keratinocytes by human papillomavirus type 16 and an activated ras oncogene. *Virology* **173** 767–771

Dyson N, Howley PM, Münger K and Harlow E (1989a) The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243** 934–937

Dyson N, Buchkovich K, Whyte P and Harlow E (1989b) The cellular 107K protein that binds to adenovirus E1A also associates with the large T antigens of SV40 and JC virus. *Cell* **58** 249–255

Edmonds C and Vousden KH (1989) A point mutational analysis of human papillomavirus type 16 E7 protein. *Journal of Virology* **63** 2650–2656

Ewen ME, Ludlow JW, DeCaprio JA *et al* (1989) An N-terminal transformation-governing sequence of SV40 large T antigen contributes to the binding of both $p110^{Rb}$ and a second cellular protein, p120. *Cell* **58** 257–267

Firzlaff JM, Galloway DA, Eisenman RN and Lüscher B (1989) The E7 protein of human papillomavirus type 16 is phosphorylated by casein kinase II. *New Biologist* **1** 44–53

Firzlaff JM, Lüscher B and Eisenman RN (1991) Negative charge at the casein kinase II phosphorylation site is important for transformation but not for Rb protein binding by the E7 protein of the human papillomavirus type 16. *Proceedings of the National Academy of Sciences of the USA* **88** 5187–5191

Goldstein D and Schlegel R (1990) The E5 oncoprotein of bovine papillomavirus binds to a 16 kd cellular protein. *EMBO Journal* **9** 137–146

Green MR (1989) When the products of oncogenes and antioncogenes meet. *Cell* **56** 1–3

Grossman SR and Laimins L (1989) E6 protein of human papilllomavirus type 18 binds zinc. *Oncogene* **4** 1089–1093

Grossman SR, Mora R and Laimins L (1989) Intracellular localization and DNA-binding properties of human papillomavirus type 18 E6 protein expressed with a baculovirus vector. *Journal of Virology* **63** 366–374

Halbert CL and Galloway D (1988) Identification of the E5 open reading frame of human papillomavirus type 16. *Journal of Virology* **62** 1071–1075

Harlow E, Whyte P, Franza BR and Schley C (1986) Association of adenovirus early region 1A proteins with cellular polypeptides. *Molecular and Cellular Biology* **6** 1579–1589

Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR and Schiller JT (1989) HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO Journal* **8** 3905–3910

Hurlin PJ, Kaur P, Smith PP, Perez-Reyes N, Blanton RA and McDougall JK (1991) Progression of human papillomavirus type 18-immortalized human keratinocytes to a malignant phenotype. *Proceedings of the National Academy of Sciences of the USA* **88** 571–574

Imai Y, Tsunokawa Y, Sugimura T and Terada M (1989) Purification and DNA-binding

properties of human papillomavirus type 16 E6 protein expressed in Escherichia coli. *Biochemical and Biophysical Research Communications* **164** 1402–1410

Jones RE, Wegrzyn RJ, Patrick DR *et al* (1990) Identification of HPV-16 E7 peptides that are potent antagonists of E7 binding to the retinoblastoma suppressor protein. *Journal of Biological Chemistry* **265** 12782–12785

Kalderon D and Smith AE (1984) In vitro mutagenesis of a putative DNA binding domain of SV40 large-T. *Virology* **139** 109–137

Kanda T, Watanabe S and Yoshiike K (1988) Immortalization of primary rat cells by human papillomavirus type 16 subgenomic fragments controlled by the SV40 promoter. *Virology* **165** 321–325

Kaur P and McDougall JK (1988) Characterisation of primary human keratinocytes transformed by human papillomavirus type 18. *Journal of Virology* **62** 1917–1924

Koi M, Morita H, Yamada H, Satoh H, Barrett JC and Oshimura H (1989) Normal human chromosome 11 suppresses tumorigenicity of human cervical tumor cell line SiHa. *Molecular Carcinogenesis* **2** 12–21

Lambert PF (1991) Papillomavirus DNA replication. *Journal of Virology* **65** 3417–3420

Lamberti C, Morrissey LC, Grossman SR and Androphy EJ (1990) Transcriptional transactivation by the human papillomavirus E6 zinc finger oncoprotein. *EMBO Journal* **9** 1907–1913

Lane DP and Crawford LV (1979) T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278** 261–263

Larose A, Dyson N, Sullivan M, Harlow E and Bastin M (1991) Polyomavirus large T mutants affected in retinoblastoma protein binding are defective in immortalization. *Journal of Virology* **65** 2308–2313

Lillie JW, Lowenstein PM, Green MR and Green M (1987) Functional domains of adenovirus type 5 E1A proteins. *Cell* **50** 1091–1100

Linzer DIH and Levine AJ (1979) Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17** 43–52

Ludlow JW, DeCaprio JA, Huang C-M, Lee W-H, Paucha E and Livingston DM (1989) SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma gene product family. *Cell* **56** 57–65

Mallon RG, Wojciechowicz D and Defendi V (1987) DNA-binding activity of papillomavirus proteins. *Journal of Virology* **61** 1655–1660

Martin P, Vass WC, Schiller JT, Lowy DR and Velu TJ (1989) The bovine papillomavirus E5 transforming protein can stimulate the transforming activity of EGF and CSF-1 receptors. *Cell* **59** 21–32

Matlashewski G, Banks L, Pim D and Crawford L (1986) Analysis of human p53 proteins and mRNA levels in normal and transformed cells. *European Journal of Biochemistry* **154** 665–672

Matlashewski G, Schneider J, Banks L, Jones N, Murray A and Crawford L (1987) Human papillomavirus type 16 cooperates with activated ras in transforming primary cells. *EMBO Journal* **6** 1741–1746

Matsukura T, Kanda T, Furuno A, Yoshikawa H, Kawana T and Yoshiike K (1986) Cloning of monomeric human papillomavirus type 16 integrated within cell DNA from a cervical carcinoma. *Journal of Virology* **58** 979–982

Matsukura T, Koi S and Sugase M (1989) Both episomal and integrated forms of human papillomavirus type 16 are involved in invasive cervical cancers. *Virology* **172** 63–72

McBride AA, Spalholz BA, Lambert PF and Howley PM (1989) Functional domains of the papillomavirus E2 proteins, In: Villareal LP (ed). *Common Mechanisms of Transformation by Small DNA Tumor Viruses*, pp 115–126, ASM Publications, Washington DC

Mihara K, Cao X-R, Yen A *et al* (1989) Cell-cycle dependent regulation of phosphorylation of the human retinoblastoma gene product. *Science* **246** 1300–1303

Miyaska M, Takami Y, Inoue H and Hakura A (1991) Rat embryo fibroblast cells suppress trans-

formation by the E6 and E7 genes of human papillomavirus type 16 in somatic hybrid cells. *Journal of Virology* **65** 479–482

Moran E and Mathews MB (1987) Multiple domains in the adenovirus E1A gene. *Cell* **48** 177–178

Moran E and Zerler B (1988) Interactions between cell growth-regulating domains in the products of the adenovirus E1A oncogene. *Molecular and Cellular Biology* **8** 1756–1764

Mori N, Yokota J, Oshimura M *et al* (1989) Concordant deletions of chromosome 3p and loss of heterozygosity for chromosome 13 and 17 in small cell lung carcinoma. *Cancer Research* **49** 5130–5135

Moses HL, Yang EY and Pietenpol JA (1990) TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* **63** 245–247

Mueller C, Graessmann A and Graessmann M (1978) Mapping of early SV40-specific functions by microinjection of different early viral DNA fragments. *Cell* **15** 579–585

Münger K, Werness BA, Dyson N, Phelps WC, Harlow E and Howley PM (1989a) Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO Journal* **8** 4099–4105

Münger K, Phelps WC, Bubb V, Howley PM and Schlegel R (1989b) The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *Journal of Virology* **63** 4417–4421

Münger K, Yee CL, Phelps WC, Pietenpol JA, Moses HL and Howley PM (1991) Biochemical and biological differences between E7 oncoproteins of the high and low risk HPV types are determined by aminoterminal sequences. *Journal of Virology* **65** 3943–3948

Nevins JR (1989) Mechanisms of viral-mediated trans-activation of transcription. *Advances in Virus Research* **37** 35–87

Oren M, Maltzman W and Levine AJ (1981) Post-translational regulation of the 54 K cellular tumor antigen in normal and transformed cells. *Molecular and Cellular Biology* **1** 101–110

Pecoraro G, Lee M, Morgan D and Defendi V (1991) Evolution of in vitro transformation and tumorigenesis of HPV 16 and HPV 18 immortalized primary cervical epithelial cells. *American Journal of Pathology* **173** 1–8

Petti L, Nilson LA and DiMaio D (1991) Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. *EMBO Journal* **10** 845–855

Phelps WC and Howley PM (1987) Transcriptional trans-activation by the human papillomavirus type 16 E2 gene product. *Journal of Virology* **61** 1630–1638

Phelps WC, Yee CL, Münger K and Howley PM (1988a) The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to adenovirus E1a. *Cell* **53** 539–547

Phelps WC, Yee CL, Münger K and Howley PM (1988b) Functional and sequence similarities between HPV16 E7 and adenovirus E1A. *Current Topics in Microbiology and Immunology* **144** 153–166

Phelps WC, Münger K, Yee CL and Howley, PM (1990) Site directed mutagenesis of the HPV16 E7 gene, In: Howley PM and Broker T (eds). *UCLA Symposia on Molecular and Cellular Biology*, new series vol 124: *Papillomaviruses*, pp 305–311, AR Liss Inc, New York

Phelps WC, Bagchi S, Barnes JA *et al* (1991) Analysis of *trans*-activation by HPV16 E7 and adenovirus 12S E1A suggests a common mechanism. *Journal of Virology* **65** 6922–6930

Pietenpol JA, Holt JT, Stein RW and Moses HL (1990a) Transforming growth factor β1 suppression of c-*myc* gene transcription: role in inhibition of keratinocyte proliferation. *Proceedings of the National Academy of Sciences of the USA* **87** 3758–3762

Pietenpol JA, Stein RW, Moran E *et al* (1990b) TGF-β1 inhibition of transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* **61** 777–785

Pirisi L, Yasumoto S, Fellerey M, Doninger JK and DiPaolo JA (1987) Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *Journal of Virology* **61** 1061–1066

Reich NC, Oren M and Levine AJ (1983) Two distinct mechanisms regulate the levels of a cellular tumor antigen. *Molecular and Cellular Biology* **3** 2134–2150

Riou G, Favre M, Jeannel DJ, Bourhis J, Le Doussal V and Orth G (1990) Association between poor prognosis in early-stage invasive cervical carcinomas and non-detection of HPV DNA. *Lancet* **335** 1171–1174

Romanczuk H, Thierry F and Howley PM (1990) Mutational analysis of cis elements involved in E2 modulation of human papillomavirus type 16 P_{97} and type 18 P_{105} promoters. *Journal of Virology* **64** 2849–2859

Rösl F, Dürst M and zur Hausen H (1988) Selective suppression of human papillomavirus transcription in non-tumorigenic cells by 5-azacytidine. *EMBO Journal* **7** 1321–1327

Sarnow P, Ho YS, Williams J and Levine AJ (1982) Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54kd cellular protein in transformed cells. *Cell* **28** 387–394

Sato H, Watanabe S, Furuno A and Yoshiike K (1989a) Human papillomavirus type 16 E7 protein expressed in Escherichia coli and monkey COS-1 cells: immunofluorescence detection of the nuclear E7 protein. *Virology* **170** 311–315

Sato H, Furuno A and Yoshiike K (1989b) Expression of human papillomavirus type 16 E7 gene induces DNA synthesis in rat 3Y1 cell. *Virology* **168** 195–199

Saxon PJ, Srivatsan ES and Stanbridge EJ (1986) Introduction of chromosome 11 via microcell transfer control tumorigenic expression of HeLa cells. *EMBO Journal* **5** 3461–3466

Scheffner M, Werness BA, Huibregtse JM, Levine AJ and Howley PM (1990) The E6 oncoprotein encoded by the human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63** 1129–1136

Scheffner M, Münger K, Byrne JC and Howley PM (1991) The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proceedings of the National Academy of Sciences of the USA* **88** 5523–5527

Schlegel R, Phelps WC, Zhang Y-L and Barbosa M (1988) Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. *EMBO Journal* **7** 3181–3187

Schwarz E, Freese UK, Gissmann L *et al* (1985) Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* **314** 111–114

Smits PHM, Smits HL, Jebbink MF and ter Schegget J (1990) The short arm of chromosome 11 likely is involved in the regulation of the human papillomavirus type 16 early enhancer-promoter and in the suppression of the transforming activity of the viral DNA. *Virology* **176** 158–165

Smotkin D and Wettstein FO (1986) Transcription of human papillomavirus type 16 early genes in cervical cancer and a cervical cancer derived cell line and identification of the E7 protein. *Proceedings of the National Academy of Sciences of the USA* **83** 4680–4684

Smotkin D, Prokoph H and Wettstein FO (1989) Oncogenic and nononcogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. *Journal of Virology* **63** 1441–1447

Stanbridge E, Dez CJ, Dorsen C-J *et al* (1982) Human cell hybrids: analysis of transformation and tumorigenicity. *Science* **215** 252

Storey A, Pim D, Murray A, Osborn K, Banks L and Crawford L (1988) Comparison of the in vitro transforming activities of human papillomavirus types. *EMBO Journal* **7** 1815–1820

Storey A, Osborn K and Crawford L (1990) Co-transformation by human papillomavirus types 6 and 11. *Journal of General Virology* **71** 165–171

Tanaka A, Noda T, Yajima H, Hatanaka M and Ito Y (1989) Identification of a transforming gene of human papillomavirus type 16. *Journal of Virology* **63** 1465–1469

Thierry F and Yaniv M (1987) The BPV-1 E2 trans-acting protein can be either an activator or repressor of the HPV-18 regulatory region. *EMBO Journal* **6** 3391–3397

Thierry F and Howley PM (1991) Functional analysis of E2-mediated repression of the HPV18 P_{105} promoter. *New Biologist* **3** 90–100

Vessey MP (1986) Epidemiology of cervical cancer: role of hormonal factors, cigarette smoking and occupation. *Banbury Report* **21** 29–43

Vousden KH, Doninger J, DiPaolo JA and Lowy DR (1988) The E7 open reading frame of human papillomavirus type 16 encodes a transforming gene. *Oncogene Research* **3** 167–175

Watanabe S and Yoshiike K (1988) Transformation of rat 3Y1 cells by human papillomavirus type 18 DNA. *International Journal of Cancer* **41** 896–900

Watanabe S, Kanda T and Yoshiike K (1989) Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. *Journal of Virology* **63** 965–969

Watanabe S, Kanda T, Sato H, Furuno A and Yoshiike K (1990) Mutational analysis of human papillomavirus type 16 E7 functions. *Journal of Virology* **64** 207–214

Werness BA, Levine AJ and Howley PM (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248** 76–79

Whyte P, Ruley HE and Harlow E (1988a) Two regions of the adenovirus early region 1A proteins are required for transformation. *Journal of Virology* **62** 257–265

Whyte P, Buchkovich KJ, Horowitz JM *et al* (1988b) Association between an oncogene and an antioncogene: the adenovirus E1a proteins bind to the retinoblastoma gene product. *Nature* **334** 124–129

Whyte P, Williamson NM and Harlow E (1989) Cellular targets for transformation by the adenovirus E1A proteins. *Cell* **56** 67–75

Yee S and Branton PE (1985) Detection of cellular proteins associated with human adenovirus type 5 early region E1A polypeptides. *Virology* **147** 142–153

Yokota J, Wada M, Shimosato Y, Terada M and Sugimura T (1987) Loss of heterozygosity on chromosomes 3, 13, and 17 in small-cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. *Proceedings of the National Academy of Sciences of the USA* **84** 9252–9256

Yokota J, Tsukada Y, Nakajima T *et al* (1989) Loss of heterozygosity on the short arm of chromosome 3 in carcinoma of the uterine cervix. *Cancer Research* **49** 3598–3601

zur Hausen H and Schneider A (1987) The role of papillomaviruses in human anogenital cancers, In: Salzman N and Howley PM (eds). *The Papovaviridae* vol 2 pp 245–263, Plenum Press, New York

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